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【Name of Document】 Claims 1

【Name of Document】 Specification 1

【Name of Document】 Drawings 1

【Name of Document】 Abstract 1

【General Power of Attorney Number】 0216136

[Document Name] Claims

[Claim 1]

An antibody comprising a single-chain polypeptide having binding activity against TPO receptor (Mpl), wherein said antibody comprises two heavy chain variable regions and two light chain variable regions.

[Claim 2]

The antibody of claim 1, wherein the two heavy chain variable regions and the two light chain variable regions are arranged in the order of heavy chain variable region, light chain variable region, heavy chain variable region, and light chain variable region from the N terminus of the single-chain polypeptide.

[Claim 3]

The antibody of claim 1 or 2, wherein the two heavy chain variable regions and the two light chain variable regions are linked by linkers.

[Claim 4]

The antibody of claim 3, wherein the linkers comprise 15 amino acids.

[Claim 5]

A chimeric antibody that binds to Mpl.

[Claim 6]

The antibody of claim 5, which is a humanized antibody.

[Claim 7]

The antibody of claim 5 or 6, which is a minibody.

[Claim 8]

An antibody that binds to soluble Mpl.

[Claim 9]

An antibody that binds to human Mpl and monkey Mpl.

[Claim 10]

An antibody having agonistic activity against human Mpl and monkey Mpl.

[Claim 11]

An antibody whose binding activity to soluble Mpl is $KD = 10^{-6}$ M or lower.

[Claim 12]

An antibody whose binding activity to soluble Mpl is $KD = 10^{-7}$ M or lower.

[Claim 13]

An antibody whose binding activity to soluble Mpl is $KD = 10^{-8}$ M or lower.

[Claim 14]

An antibody whose TPO agonistic activity is $EC50 = 100$ nM or lower.

[Claim 15]

An antibody whose TPO agonistic activity is $EC_{50} = 30$ nM or lower.

[Claim 16]

An antibody whose TPO agonistic activity is $EC_{50} = 10$ nM or lower.

[Claim 17]

5 An antibody which comprises a heavy chain variable region, wherein said heavy chain variable regions comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any one of:

[1] SEQ ID NOs: 3, 4, and 5

[2] SEQ ID NOs: 6, 7, and 8

10 [3] SEQ ID NOs: 9, 10, and 11

[4] SEQ ID NOs: 12, 13, and 14

[5] SEQ ID NOs: 15, 16, and 17

[6] SEQ ID NOs: 18, 19, and 20

[7] SEQ ID NOs: 21, 22, and 23

15 [8] SEQ ID NOs: 24, 25, and 26

[9] SEQ ID NOs: 27, 28, and 29

[10] SEQ ID NOs: 30, 31, and 32

[11] SEQ ID NOs: 33, 34, and 35

[12] SEQ ID NOs: 36, 37, and 38

20 [13] SEQ ID NOs: 39, 40, and 41

[14] SEQ ID NOs: 42, 43, and 44

[15] SEQ ID NOs: 45, 46, and 47

[16] SEQ ID NOs: 48, 49, and 50

[17] SEQ ID NOs: 51, 52, and 53

25 [18] SEQ ID NOs: 54, 55, and 56

[19] SEQ ID NOs: 57, 58, and 59

[Claim 18]

An antibody which comprises a light chain variable region, wherein said light chain variable region comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any
30 one of:

[1] SEQ ID NOs: 60, 61, and 62

[2] SEQ ID NOs: 63, 64, and 65

[3] SEQ ID NOs: 66, 67, and 68

[4] SEQ ID NOs: 69, 70, and 71

35 [5] SEQ ID NOs: 72, 73, and 74

[6] SEQ ID NOs: 75, 76, and 77

[7] SEQ ID NOs: 78, 79, and 80

[8] SEQ ID NOs: 81, 82, and 83

[9] SEQ ID NOs: 84, 85, and 86

[10] SEQ ID NOs: 87, 88, and 89

5 [11] SEQ ID NOs: 90, 91, and 92

[12] SEQ ID NOs: 93, 94, and 95

[13] SEQ ID NOs: 96, 97, and 98

[14] SEQ ID NOs: 99, 100, and 101

[15] SEQ ID NOs: 102, 103, and 104

10 [16] SEQ ID NOs: 105, 106, and 107

[17] SEQ ID NOs: 108, 109, and 110

[18] SEQ ID NOs: 111, 112, and 113

[19] SEQ ID NOs: 114, 115, and 116.

[Claim 19]

15 An antibody that comprises a heavy chain variable region and a light chain variable region of any one of:

[1] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 3, 4, and 5, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting
20 of SEQ ID NOs: 60, 61, and 62;

[2] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 6, 7, and 8, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 63, 64, and 65;

25 [3] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 9, 10, and 11, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 66, 67, and 68;

[4] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising
30 the amino acid sequences consisting of SEQ ID NOs: 12, 13, and 14, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 69, 70, and 71;

[5] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 15, 16, and 17, and a light chain variable
35 region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 72, 73, and 74;

[6] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 18, 19, and 20, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 75, 76, and 77;

5 [7] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 21, 22, and 23, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 78, 79, and 80;

10 [8] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 24, 25, and 26, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 81, 82, and 83;

15 [9] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 27, 28, and 29, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 84, 85, and 86;

20 [10] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 30, 31, and 32, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 87, 88, and 89;

[11] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 33, 34, and 35, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 90, 91, and 92;

25 [12] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 36, 37, and 38, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 93, 94, and 95;

30 [13] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 39, 40, and 41, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 96, 97, and 98;

35 [14] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 42, 43, and 44, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 99, 100, and 101;

[15] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 45, 46, and 47, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 102, 103, and 104;

5 [16] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 48, 49, and 50, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 105, 106, and 107;

10 [17] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 51, 52, and 53, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 108, 109, and 110;

15 [18] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 54, 55, and 56, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 111, 112, and 113;

20 [19] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 57, 58, and 59, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 114, 115, and 116.

[Claim 20]

An antibody that comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 118.

[Claim 21]

25 An antibody that comprises a light chain variable region comprising the amino acid sequence of SEQ ID NO: 120.

[Claim 22]

30 An antibody that comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 118 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 120.

[Claim 23]

An antibody comprising the amino acid sequence of SEQ ID NO: 122.

[Claim 24]

An antibody comprises the amino acid sequence of SEQ ID NO: 2.

35 [Claim 25]

An antibody having an activity equivalent to that of an antibody of any one of claims 17

to 24, wherein said antibody comprises the amino acid sequence set forth in any one of claims 17 to 24, in which one or more amino acids have been substituted, deleted, added and/or inserted.

[Claim 26]

5 An antibody that recognizes an epitope recognized by an antibody of any one of claims 17 to 25.

[Claim 27]

An antibody that recognizes the region of amino acids 26 to 274 of human Mpl.

[Claim 28]

An antibody of any one of claims 1 to 27, which has TPO agonistic activity.

10 [Claim 29]

A polynucleotide encoding an antibody of any one of claims 1 to 28.

[Claim 30]

15 A polynucleotide hybridizing to the polynucleotide of claim 29 under stringent conditions, wherein said polynucleotide encodes an antibody having activity equivalent to that of an antibody of any one of claims 1 to 28.

[Claim 31]

A vector comprising the polynucleotide of claim 29 or 30.

[Claim 32]

A host cell that carries the polynucleotide of claim 29 or 30, or the vector of claim 31.

20 [Claim 33]

A pharmaceutical composition comprising an antibody of any one of claims 1 to 28.

[Document Name] Specification

[Title of the Invention] ANTI-MPL ANTIBODIES

[Technical Field]

5 [0001]

The present invention relates to anti-Mpl antibodies.

[Background of the Invention]

[0002]

10 Thrombopoietin (TPO) is a factor that enhances the differentiation and maturation of megakaryocytes (platelet precursor cells) from hemopoietic stem cells into platelets. TPO also functions as a cytokine with an important role in the regulation of platelet number. TPO is converted into its active form through the cleavage of a TPO precursor comprising 353 amino acids.

15 Mpl is a TPO receptor, and human Mpl molecules are known to exist in two forms comprising 572 and 635 amino acids. The human Mpl gene sequence has already been analyzed (see Non-patent Document 1 and GenBank accession No. NM_005373).

20 Most cytokine receptors dimerize upon ligand binding, and transduce signals into cells. It has been reported that TPO similarly binds to its own specific receptor MPL, which leads to dimerization of the receptor, thereby transducing signals into cells and exerting physiological effects (see Non-patent Document 2).

Antibodies exhibiting agonistic activity have been reported among those antibodies that bind to receptors having the above features.

25 For example, an antibody against the erythropoietin (EPO) receptor has been reported to substitute for erythropoietin function. The monovalent form (Fab) of the antibody is capable of binding to the EPO receptor but is unable to transduce signals. Thus, dimerization of the erythropoietin receptor via bivalent binding is assumed to be essential for signal transduction (see Non-patent Document 3).

30 Antibodies that bind to Mpl and exhibit TPO agonistic activity have also been reported (see Non-patent Documents 4 and 5). This suggests that receptor dimerization is induced upon binding of a bivalent antibody with regards to MPL as well.

Meanwhile, a single-chain antibody (scFv) has been reported to exhibit TPO agonistic activity (see Patent Document 1). However, it has been revealed that, the underlying mechanism of scFv exhibiting TPO agonistic activity is that a part of scFv dimerizes (diabody) and this diabody becomes the actual active unit (see Patent Documents 2 to 4).

35 [0003]

[Patent Document 1] US Patent No. 6342220

[Patent Document 2] WO 01/79494

[Patent Document 3] WO 02/33072

[Patent Document 4] WO 02/33073

[Non-patent Document 1] Palacios *et al.*, 1985, Cell, 41, 727-734

5 [Non-patent Document 2] Souyri *et al.*, 1990, Cell, Vol.63, 1137-1147

[Non-patent Document 3] Elliott, S. *et al.*, 1996, J. Biol. Chem., 271(40), 24691-24697

[Non-patent Document 4] Abe *et al.*, 1998, Immunol. Lett., 61, 73-78

[Non-patent Document 5] Bijia Deng *et al.*, 1998, Blood, 92, 1981-1988

[Disclosure of the Invention]

10 [Problems to be Solved by the Invention]

[0004]

The present invention was achieved in view of the above circumstances. An objective of the present invention is to provide novel anti-Mpl antibodies having TPO agonistic activity.

[Means for Solving the Problems]

15 [0005]

The present inventors performed exhaustive research to solve the above objective. The present inventors prepared and purified anti-human Mpl antibody VB22B, and established a single-chain antibody expression system using genetic engineering techniques. Specifically, the variable region of anti-human Mpl antibody was first cloned, and a diabody expression vector

20 pCXND3-VB22B db for the anti-human Mpl antibody was prepared. This pCXND3-VB22B db vector was then used to generate an expression vector pCXND3-VB22B sc(Fv)₂ for anti-human Mpl antibody sc(Fv)₂. Anti-human Mpl sc(Fv)₂ was transiently expressed in CHO-DG44 cells using the expression vector pCXND3-VB22B sc(Fv)₂, and then purified from the culture supernatant. In control experiments, VB22B diabody was transiently expressed in

25 COS7 cells using the above pCXND3-VB22B db vector, and then purified from the culture supernatant.

[0006]

In addition, VB22B diabody and VB22B sc(Fv)₂ were evaluated for their TPO-like agonistic activities. The results showed that VB22B diabody and VB22B sc(Fv)₂ exhibit higher

30 agonistic activities compared to VB22B IgG, and thus activities equivalent to or higher than that of the natural ligand, human TPO.

[0007]

More specifically, the present invention relates to:

(1) an antibody comprising a single-chain polypeptide having binding activity against

35 TPO receptor (Mpl), wherein said antibody comprises two heavy chain variable regions and two light chain variable regions;

(2) the antibody of (1), wherein the two heavy chain variable regions and the two light chain variable regions are arranged in the order of heavy chain variable region, light chain variable region, heavy chain variable region, and light chain variable region from the N terminus of the single-chain polypeptide;

5 (3) the antibody of (1) or (2), wherein the two heavy chain variable regions and the two light chain variable regions are linked by linkers;

(4) the antibody of (3), wherein the linkers comprise 15 amino acids;

(5) a chimeric antibody that binds to Mpl;

(6) the antibody of (5), which is a humanized antibody;

10 (7) the antibody of (5) or (6), which is a minibody;

(8) an antibody that binds to soluble Mpl;

(9) an antibody that binds to human Mpl and monkey Mpl;

(10) an antibody having agonistic activity against human Mpl and monkey Mpl;

(11) an antibody whose binding activity to soluble Mpl is $KD = 10^{-6}$ M or lower;

15 (12) an antibody whose binding activity to soluble Mpl is $KD = 10^{-7}$ M or lower;

(13) an antibody whose binding activity to soluble Mpl is $KD = 10^{-8}$ M or lower;

(14) an antibody whose TPO agonistic activity is $EC50 = 100$ nM or lower;

(15) an antibody whose TPO agonistic activity is $EC50 = 30$ nM or lower;

(16) an antibody whose TPO agonistic activity is $EC50 = 10$ nM or lower;

20 (17) an antibody which comprises a heavy chain variable region, wherein said heavy chain variable regions comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any one of:

[1] SEQ ID NOs: 3, 4, and 5

[2] SEQ ID NOs: 6, 7, and 8

25 [3] SEQ ID NOs: 9, 10, and 11

[4] SEQ ID NOs: 12, 13, and 14

[5] SEQ ID NOs: 15, 16, and 17

[6] SEQ ID NOs: 18, 19, and 20

[7] SEQ ID NOs: 21, 22, and 23

30 [8] SEQ ID NOs: 24, 25, and 26

[9] SEQ ID NOs: 27, 28, and 29

[10] SEQ ID NOs: 30, 31, and 32

[11] SEQ ID NOs: 33, 34, and 35

[12] SEQ ID NOs: 36, 37, and 38

35 [13] SEQ ID NOs: 39, 40, and 41

[14] SEQ ID NOs: 42, 43, and 44

[15] SEQ ID NOs: 45, 46, and 47

[16] SEQ ID NOs: 48, 49, and 50

[17] SEQ ID NOs: 51, 52, and 53

[18] SEQ ID NOs: 54, 55, and 56

5 [19] SEQ ID NOs: 57, 58, and 59;

(18) an antibody which comprises a light chain variable region, wherein said light chain variable region comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any one of:

[1] SEQ ID NOs: 60, 61, and 62

10 [2] SEQ ID NOs: 63, 64, and 65

[3] SEQ ID NOs: 66, 67, and 68

[4] SEQ ID NOs: 69, 70, and 71

[5] SEQ ID NOs: 72, 73, and 74

[6] SEQ ID NOs: 75, 76, and 77

15 [7] SEQ ID NOs: 78, 79, and 80

[8] SEQ ID NOs: 81, 82, and 83

[9] SEQ ID NOs: 84, 85, and 86

[10] SEQ ID NOs: 87, 88, and 89

[11] SEQ ID NOs: 90, 91, and 92

20 [12] SEQ ID NOs: 93, 94, and 95

[13] SEQ ID NOs: 96, 97, and 98

[14] SEQ ID NOs: 99, 100, and 101

[15] SEQ ID NOs: 102, 103, and 104

[16] SEQ ID NOs: 105, 106, and 107

25 [17] SEQ ID NOs: 108, 109, and 110

[18] SEQ ID NOs: 111, 112, and 113

[19] SEQ ID NOs: 114, 115, and 116;

(19) an antibody that comprises a heavy chain variable region and a light chain variable region of any one of:

30 [1] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 3, 4, and 5, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 60, 61, and 62;

35 [2] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 6, 7, and 8, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting

of SEQ ID NOs: 63, 64, and 65;

[3] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 9, 10, and 11, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 66, 67, and 68;

[4] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 12, 13, and 14, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 69, 70, and 71;

[5] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 15, 16, and 17, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 72, 73, and 74;

[6] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 18, 19, and 20, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 75, 76, and 77;

[7] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 21, 22, and 23, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 78, 79, and 80;

[8] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 24, 25, and 26, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 81, 82, and 83;

[9] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 27, 28, and 29, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 84, 85, and 86;

[10] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 30, 31, and 32, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 87, 88, and 89;

[11] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 33, 34, and 35, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting

of SEQ ID NOs: 90, 91, and 92;

[12] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 36, 37, and 38, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 93, 94, and 95;

[13] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 39, 40, and 41, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 96, 97, and 98;

[14] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 42, 43, and 44, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 99, 100, and 101;

[15] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 45, 46, and 47, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 102, 103, and 104;

[16] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 48, 49, and 50, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 105, 106, and 107;

[17] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 51, 52, and 53, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 108, 109, and 110;

[18] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 54, 55, and 56, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 111, 112, and 113;

[19] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 57, 58, and 59, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 114, 115, and 116;

(20) an antibody that comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 118;

(21) an antibody that comprises a light chain variable region comprising the amino acid

sequence of SEQ ID NO: 120;

(22) an antibody that comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 118 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 120;

5 (23) an antibody comprising the amino acid sequence of SEQ ID NO: 122;

(24) an antibody comprises the amino acid sequence of SEQ ID NO: 2;

(25) an antibody having an activity equivalent to that of an antibody of any one of (17) to (24), wherein said antibody comprises the amino acid sequence set forth in any one of (17) to (24), in which one or more amino acids have been substituted, deleted, added and/or inserted;

10 (26) an antibody that recognizes an epitope recognized by an antibody of any one of (17) to (25);

(27) an antibody that recognizes the region of amino acids 26 to 274 of human Mpl;

(28) an antibody of any one of (1) to (27), which has TPO agonistic activity;

(29) a polynucleotide encoding an antibody of any one of (1) to (28);

15 (30) a polynucleotide hybridizing to the polynucleotide of (29) under stringent conditions, wherein said polynucleotide encodes an antibody having activity equivalent to that of an antibody of any one of (1) to (28);

(31) a vector comprising the polynucleotide of (29) or (30);

(32) a host cell that carries the polynucleotide of (29) or (30), or the vector of (31); and

20 (33) a pharmaceutical composition comprising an antibody of any one of (1) to (28).

[Effects of the Invention]

[0008]

Recombinant human TPOs have been tested as therapeutic agents for chemotherapy-induced thrombocytopenia in various clinical trials. In these trials, a major
25 problem that was reported was the production of anti-TPO antibody in TPO-treated patients (Junzhi Li, *et al.*, Blood (2001) 98: 3241-324; Saroj Vandhan-Raj, *et al.*, Ann. Intern. Med. (2000) 132: 364-368). Specifically, production of neutralizing antibodies that inhibit endogenous TPO activity, and the resulting onset of thrombocytopenia, were reported. Administration of agonistic minibodies (low molecular weight antibodies) against anti-TPO
30 receptor of the present invention does not induce the production of antibodies against endogenous TPO. Furthermore, since antibody miniaturization results in increased specific activity and short half-life in blood, the effective concentration in blood can be easily regulated, presenting a further clinical advantage. Accordingly, the present antibodies are expected to be therapeutic agents for thrombocytopenia which are more effective than naturally-occurring TPO
35 or its agonistic antibodies. Furthermore, since minibodies are not glycosylated, expression systems for expressing the recombinant proteins are not limited, and minibodies can be produced

in any expression system such as cell lines from mammals, yeast, insect cells, and *E. coli*. Since binding specificity against mutant TPO receptor is different from that of TPO, minibodies are expected to bind specific mutants and show agonistic activity against mutated TPO receptor detected in the CAMT patients, who genetically have mutated TPO receptor and develop thrombocytopenia.

[Best Mode for Carrying Out the Invention]

[0009]

The present invention provides antibodies that bind to the TPO receptor (Mpl).

The antibodies of the present invention comprise all types of antibodies, including antibodies with modified amino acid sequences, such as minibodies, humanized antibodies, and chimeric antibodies; antibodies that have been modified by binding with other molecules (for example, polymers such as polyethylene glycol); and antibodies whose sugar chains have been modified.

It is preferable that the antibodies of the present invention have agonistic activity against Mpl.

[0010]

In a preferred embodiment, the antibodies of the present invention comprise, for example, minibodies.

The minibodies comprise antibody fragments lacking portions of the whole antibody (for example, whole IgG). The minibodies are not particularly limited as long as they have binding activity to their antigens. The minibodies of the present invention have markedly higher activities compared to their corresponding whole antibodies. There are no particular limitations on the antibody fragments of the present invention as long as they are portions of the whole antibody, and preferably contain heavy chain variable regions (VH) and/or light chain variable regions (VL). The amino acid sequences of VH or VL may contain substitutions, deletions, additions and/or insertions. Furthermore, the antibody fragment may also lack portions of VH or/and VL, as long as it has binding ability to its antigen. In addition, the variable regions may be chimerized or humanized. Such antibody fragments include, for example, Fab, Fab', F(ab')₂, and Fv. An example of a minibody includes Fab, Fab', F(ab')₂, Fv, scFv (single-chain Fv), diabody, and sc(Fv)₂ (single-chain (Fv)₂).

[0011]

Herein, an "Fv" fragment is the smallest antibody fragment and contains a complete antigen recognition site and a binding site. The "Fv" fragment is a dimer (VH-VL dimer) in which a single VH and a single VL are strongly linked by a non-covalent bond. The three complementarity-determining regions (CDRs) of each of the variable regions interact with each other to form an antigen-binding site on the surface of the VH-VL dimer. Six CDRs confer the

antigen-binding site of an antibody. However, a single variable region (or a half of Fv containing only three CDRs specific to an antigen) alone is also capable of recognizing and binding an antigen although its affinity is lower than the affinity of the entire binding site.

[0012]

5 scFv contains the VH and VL regions of an antibody, and these regions exist on a single polypeptide chain. Generally, an Fv polypeptide further contains a polypeptide linker between VH and VL, and therefore an scFv can form a structure required for antigen binding. See, Pluckthun "The Pharmacology of Monoclonal Antibodies" Vol. 113 (Rosenburg and Moore eds. (Springer Verlag, New York, pp.269-315, 1994) for the review of scFv. In the present invention,
10 linkers are not especially limited as long as they do not inhibit expression of antibody variable regions linked at both ends of the linkers.

[0013]

The term "diabody" refers to a bivalent antibody fragment constructed by gene fusion (Holliger P *et al.*, Proc. Natl. Acad. Sci. USA (1993) 90: 6444-6448; EP 404,097; WO 93/11161
15 and others). Diabodies are dimers comprising two polypeptide chains, where each polypeptide chain comprises a VL and a VH connected with a linker short enough to prevent interaction of these two domains, for example, a linker of about five residues. The VL and VH encoded on the same polypeptide chain will form a dimer because the linker between them is too short to form a single-chain variable region fragment. As a result, the polypeptide chains form a dimer,
20 and thus the diabody has two antigen binding sites.

[0014]

sc(Fv)₂ is a single-chain minibody produced by linking two units of VH and two units of VL with linkers and such (Hudson *et al.*, J Immunol. Methods (1999) 231: 177-189). sc(Fv)₂ exhibits a particularly high agonistic activity compared to the whole antibody and other
25 minibodies. sc(Fv)₂ can be produced, for example, by linking two scFv molecules.

[0015]

In a preferable antibody, the two VH units and two VL units are arranged in the order of VH, VL, VH, and VL ([VH]-linker-[VL]-linker-[VH]-linker-[VL]) beginning from the N terminus of a single-chain polypeptide.

30 The order of the two VH units and two VL units is not limited to the above arrangement, and they may be arranged in any order. Examples of the arrangements are listed below.

[VL]-linker-[VH]-linker-[VH]-linker-[VL]

[VH]-linker-[VL]-linker-[VL]-linker-[VH]

[VH]-linker-[VH]-linker-[VL]-linker-[VL]

35 [VL]-linker-[VL]-linker-[VH]-linker-[VH]

[VL]-linker-[VH]-linker-[VL]-linker-[VH]

[0016]

The linkers to be used for linking the variable regions of an antibody comprise arbitrary peptide linkers that can be introduced by genetic engineering, synthetic linkers, and linkers disclosed in, for example, Holliger, P. *et al.*, Protein Engineering (1996) 9 (3): 299-305.

5 Peptide linkers are preferred in the present invention. There are no limitations as to the length of the peptide linkers. The length can be selected accordingly by those skilled in the art depending on the purpose, and is typically 1-100 amino acids, preferably 3-50 amino acids, more preferably 5-30 amino acids, and even more preferably 12-18 amino acids (for example, 15 amino acids).

10 [0017]

For example, such peptide linkers include:

Ser

Gly Ser

Gly Gly Ser

15 Ser Gly Gly

Gly Gly Gly Ser

Ser Gly Gly Gly

Gly Gly Gly Gly Ser

Ser Gly Gly Gly Gly

20 Gly Gly Gly Gly Gly Ser

Ser Gly Gly Gly Gly Gly

Gly Gly Gly Gly Gly Gly Ser

Ser Gly Gly Gly Gly Gly Gly

(Gly Gly Gly Gly Ser)_n

25 (Ser Gly Gly Gly Gly)_n

where n is an integer of 1 or larger. The lengths and sequences of peptide linkers can be selected accordingly by those skilled in the art depending on the purpose.

[0018]

In an embodiment of the present invention, a particularly preferable sc(Fv)₂ includes,
30 for example, the sc(Fv)₂ below.

[VH]-peptide linker (15 amino acids)-[VL]-peptide linker (15 amino acids)-[VH]-peptide linker (15 amino acids)-[VL]

[0019]

Synthetic linkers (chemical crosslinking agents) include crosslinking agents routinely
35 used to crosslink peptides, for example, N-hydroxy succinimide (NHS), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl) suberate (BS³), dithiobis(succinimidyl propionate) (DSP),

dithiobis(sulfosuccinimidyl propionate) (DTSSP), ethylene glycol bis(succinimidyl succinate) (EGS), ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS), disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo-DST), bis[2-(succinimidoxycarbonyloxy)ethyl] sulfone (BSOCOES), and bis[2-(sulfosuccinimidoxycarbonyloxy)ethyl] sulfone (sulfo-BSOCOES). These crosslinking agents are commercially available.

[0020]

In general, three linkers are required to link four antibody variable regions together. The linkers to be used may be of the same type or different types. In the present invention, a preferable minibody is a diabody, even more preferably, an $sc(Fv)_2$. Such a minibody can be prepared by treating an antibody with an enzyme, for example, papain or pepsin, to generate antibody fragments, or by constructing DNAs encoding those antibody fragments and introducing them into expression vectors, followed by expression in an appropriate host cell (see, for example, Co, M. S. *et al.*, J. Immunol. (1994) 152: 2968-2976; Better, M. and Horwitz, A. H., Methods Enzymol. (1989) 178: 476-496; Pluckthun, A. and Skerra, A., Methods Enzymol. (1989) 178: 497-515; Lamoyi, E., Methods Enzymol. (1986) 121: 652-663; Rousseaux, J. *et al.*, Methods Enzymol. (1986) 121: 663-669; Bird, R. E. and Walker, B. W., Trends Biotechnol. (1991) 9: 132-137).

An antibody having exceedingly high agonistic activity can be prepared by reducing the molecular weight of a full-length antibody, particularly by converting it into an $sc(Fv)_2$.

[0021]

In a preferred embodiment, the antibodies of the present invention comprise modified antibodies, such as chimeric antibodies and humanized antibodies that bind to Mpl. These modified antibodies can be produced by known methods.

Chimeric antibodies are antibodies prepared by combining sequences derived from different animal species, and include for example, antibodies comprising the heavy chain and light chain variable regions of a murine antibody, and the heavy chain and light chain constant regions of a human antibody. Chimeric antibodies can be prepared by known methods. For example, a DNA encoding the V region of an antibody is linked to a DNA encoding the C region of a human antibody, and the construct is inserted into an expression vector and introduced into a host to produce chimeric antibodies.

Humanized antibodies are also referred to as "reshaped human antibodies". Such a humanized antibody is obtained by transferring the complementarity-determining region (CDR) of an antibody derived from a non-human mammal, for example mouse, to the complementarity-determining region of a human antibody, and the general gene recombination procedure for this is also known (see European Patent Application No. 125023 and WO 96/02576).

Specifically, a DNA sequence designed to link a murine antibody CDR to the framework region (FR) of a human antibody can be synthesized by PCR, using primers prepared from several oligonucleotides containing overlapping portions of both CDR and FR terminal regions (see methods described in WO 98/13388).

5 The human antibody framework region to be linked by CDR is selected in order to form a favorable antigen-binding site in the complementarity-determining region. Amino acids of the framework region in the antibody variable region may be substituted, as necessary, for the complementarity-determining region of the reshaped human antibody to form a suitable antigen-binding site (Sato, K. *et al.*, Cancer Res. (1993) 53: 851-856).

10 The constant region of a human antibody is used as the C region of a chimeric antibody or humanized antibody. For example, $C\gamma 1$, $C\gamma 2$, $C\gamma 3$, and $C\gamma 4$ can be used as the H chain, and $C\kappa$ and $C\lambda$ can be used as the L chain. The human antibody C region may be modified to improve the antibody or the stability of the antibody production.

15 Generally, chimeric antibodies comprise the variable region of an antibody from a non-human mammal and the constant region derived from a human antibody. On the other hand, humanized antibodies comprise the complementarity-determining region of an antibody from a non-human mammal, and the framework region and C region derived from a human antibody.

20 In addition, after a chimeric antibody or a humanized antibody is prepared, amino acids in the variable region (for example, FR) and the constant region may be replaced with other amino acids, and such.

25 The origin of the variable regions in chimeric antibodies or that of the CDRs in humanized antibodies is not particularly limited, and may be derived from any type of animal. For example, sequences of murine antibodies, rat antibodies, rabbit antibodies, camel antibodies may be used.

 In general, it is difficult to chimerize or humanize an antibody without losing the agonistic activity of the original antibody. Nevertheless, the present invention succeeded in preparing humanized antibodies having agonistic activity equivalent to that of the original murine antibody.

30 Chimeric antibodies and humanized antibodies exhibit lower antigenicity in the human body, and thus are expected to be useful when administered to humans for therapeutic purposes.

[0022]

35 In one embodiment, the preferred antibodies of the present invention include antibodies that bind to soluble Mpl. The term "soluble Mpl" herein refers to Mpl molecules excluding those expressed on the cell membrane. A specific example of a soluble Mpl is an Mpl lacking the entire or a portion of the transmembrane domain. The transmembrane domain of human

Mpl corresponds to amino acids 492 to 513 in SEQ ID NO: 123.

An antibody that binds to soluble recombinant Mpl can be used in detailed epitope analysis and kinetic analysis of receptor-ligand binding, as well as for assessing the blood concentration and dynamic behavior of the antibody in *in vivo* tests.

5 In one embodiment, the preferred antibodies of the present invention include antibodies having binding activity against both human and monkey Mpl. Antibodies having agonistic activity to both human and monkey Mpl are expected to be highly useful since the dynamic behavior and *in vivo* effects of the antibody, which are generally difficult to determine in human body, can be examined with monkeys.

10 The present invention also provides antibodies having agonistic activity to human Mpl and monkey Mpl. Such antibodies may also have binding activity or agonistic activity against Mpl from animals other than humans and monkeys (for example, mice).

In addition, the antibodies of the present invention include antibodies with TPO agonistic activity (agonistic activity against Mpl) of $EC_{50} = 100$ nM or lower, preferably $EC_{50} =$
15 30 nM or lower, more preferably $EC_{50} = 10$ nM or lower.

The agonistic activity can be determined by methods known to those skilled in the art, for example, by the method described below.

The sequences for human Mpl (Palacios *et al.*, Cell (1985) 41: 727-734; GenBank Accession NO. NM_005373), cynomolgus monkey Mpl (SEQ ID NO: 157), and mouse Mpl (GenBank
20 Accession NO. NM_010823) are already known.

In addition, the present invention includes antibodies whose binding activities to soluble Mpl are $KD = 10^{-6}$ M or lower, preferably $KD = 10^{-7}$ M or lower, and more preferably $KD = 10^{-8}$ M or lower.

[0023]

25 In the present invention, whether the binding activity of an antibody to soluble recombinant Mpl is $KD = 10^{-6}$ M or lower can be determined by methods known to those skilled in the art. For example, the activity can be determined using surface plasmon resonance with Biacore. Specifically, soluble MPL-Fc protein is immobilized onto sensor chips. Reaction rate constant can be determined by assessing the interaction between the antibody and the
30 soluble Mpl-Fc protein. The binding activity can be evaluated by ELISA (enzyme-linked immunosorbent assays), EIA (enzyme immunoassays), RIA (radio immunoassays), or fluorescent antibody techniques. For example, in enzyme immunoassays, a sample containing a test antibody, such as purified antibody or culture supernatant of cells producing the test antibody, is added to a plate coated with an antigen to which the test antibody can bind. After incubating
35 the plate with a secondary antibody labeled with an enzyme such as alkaline phosphatase, the plate is washed and an enzyme substrate such as p-nitrophenyl phosphate is added. The

antigen-binding activity can then be evaluated by determining the absorbance.

There is no specific limitation as to the upper limit of the binding activity; for example, the upper limit may be set within a technically feasible range by those skilled in the art.

However, the technically feasible range may expand with the advancement of technology.

5 [0024]

In an embodiment, the preferred antibodies of the present invention include antibodies recognizing epitopes that are recognized by any one of the antibodies indicated in (I) to (IX) below. The antibody of any one of (I) to (IX) is preferably a minibody.

[0025]

10 (I)

Antibody comprising a VH that has CDR1, 2, and 3 comprising the amino acid sequences according to SEQ ID NOs in any one of (1) to (19) indicated below (name of each antibody and the H chain CDR contained in the antibody are indicated inside the parentheses):

- (1) SEQ ID NOs: 3, 4, and 5 (VA7: H chain CDR1, 2, and 3),
- 15 (2) SEQ ID NOs: 6, 7, and 8 (VA130: H chain CDR1, 2, and 3),
- (3) SEQ ID NOs: 9, 10, and 11 (VA259: H chain CDR1, 2, and 3),
- (4) SEQ ID NOs: 12, 13, and 14 (VB17B: H chain CDR1, 2, and 3),
- (5) SEQ ID NOs: 15, 16, and 17 (VB12B: H chain CDR1, 2, and 3),
- (6) SEQ ID NOs: 18, 19, and 20 (VB140: H chain CDR1, 2, and 3),
- 20 (7) SEQ ID NOs: 21, 22, and 23 (VB33: H chain CDR1, 2, and 3),
- (8) SEQ ID NOs: 24, 25, and 26 (VB45B: H chain CDR1, 2, and 3),
- (9) SEQ ID NOs: 27, 28, and 29 (V8B: H chain CDR1, 2, and 3),
- (10) SEQ ID NOs: 30, 31, and 32 (VB115: H chain CDR1, 2, and 3),
- (11) SEQ ID NOs: 33, 34, and 35 (V14B: H chain CDR1, 2, and 3),
- 25 (12) SEQ ID NOs: 36, 37, and 38 (V22B: H chain CDR1, 2, and 3),
- (13) SEQ ID NOs: 39, 40, and 41 (VB16: H chain CDR1, 2, and 3),
- (14) SEQ ID NOs: 42, 43, and 44 (VB157: H chain CDR1, 2, and 3),
- (15) SEQ ID NOs: 45, 46, and 47 (VB4B: H chain CDR1, 2, and 3),
- (16) SEQ ID NOs: 48, 49, and 50 (VB51: H chain CDR1, 2, and 3),
- 30 (17) SEQ ID NOs: 51, 52, and 53 (AB317: H chain CDR1, 2, and 3),
- (18) SEQ ID NOs: 54, 55, and 56 (AB324: H chain CDR1, 2, and 3),
- (19) SEQ ID NOs: 57, 58, and 59 (TA136: H chain CDR1, 2, and 3).

[0026]

(II)

- 35 Antibody comprising a VL which has CDR1, 2, and 3 comprising the amino acid sequences according to SEQ ID NOs in any one of (1) to (19) indicated below (name of each

antibody and the L chain CDR in the antibody are indicated inside the parentheses):

- (1) SEQ ID NOs: 60, 61, and 62 (VA7: L chain CDR1, 2, and 3),
- (2) SEQ ID NOs: 63, 64, and 65 (VA130: L chain CDR1, 2, and 3),
- (3) SEQ ID NOs: 66, 67, and 68 (VA259: L chain CDR1, 2, and 3),
- 5 (4) SEQ ID NOs: 69, 70, and 71 (VB17B: L chain CDR1, 2, and 3),
- (5) SEQ ID NOs: 72, 73, and 74 (VB12B: L chain CDR1, 2, and 3),
- (6) SEQ ID NOs: 75, 76, and 77 (VB140: L chain CDR1, 2, and 3),
- (7) SEQ ID NOs: 78, 79, and 80 (VB33: L chain CDR1, 2, and 3),
- (8) SEQ ID NOs: 81, 82, and 83 (VB45B: L chain CDR1, 2, and 3),
- 10 (9) SEQ ID NOs: 84, 85, and 86 (V8B: L chain CDR1, 2, and 3),
- (10) SEQ ID NOs: 87, 88, and 89 (VB115: L chain CDR1, 2, and 3),
- (11) SEQ ID NOs: 90, 91, and 92 (V14B: L chain CDR1, 2, and 3),
- (12) SEQ ID NOs: 93, 94, and 95 (VB22B: L chain CDR1, 2, and 3),
- (13) SEQ ID NOs: 96, 97, and 98 (VB16: L chain CDR1, 2, and 3),
- 15 (14) SEQ ID NOs: 99, 100, and 101 (VB157: L chain CDR1, 2, and 3),
- (15) SEQ ID NOs: 102, 103, and 104 (VB4B: L chain CDR1, 2, and 3),
- (16) SEQ ID NOs: 105, 106, and 107 (VB51: L chain CDR1, 2, and 3),
- (17) SEQ ID NOs: 108, 109, and 110 (AB317: L chain CDR1, 2, and 3),
- (18) SEQ ID NOs: 111, 112, and 113 (AB324: L chain CDR1, 2, and 3),
- 20 (19) SEQ ID NOs: 114, 115, and 116 (TA136: L chain CDR1, 2, and 3).

[0027]

(III)

Antibody comprising a VH that comprises an amino acid sequence of the SEQ ID NO in any one of (1) to (19):

- 25 (1) SEQ ID NO: 124 (VA7: VH),
- (2) SEQ ID NO: 126 (VA130: VH),
- (3) SEQ ID NO: 128 (VA259: VH),
- (4) SEQ ID NO: 130 (VB17B: VH),
- (5) SEQ ID NO: 132 (VB12B: VH),
- 30 (6) SEQ ID NO: 134 (VB140: VH),
- (7) SEQ ID NO: 136 (VB33: VH),
- (8) SEQ ID NO: 138 (VB45B: VH),
- (9) SEQ ID NO: 140 (V8B: VH),
- (10) SEQ ID NO: 142 (VB115: VH),
- 35 (11) SEQ ID NO: 144 (VB14B: VH),
- (12) SEQ ID NO: 118 (VB22B: VH),

- (13) SEQ ID NO: 146 (VB16: VH),
- (14) SEQ ID NO: 148 (VB157: VH),
- (15) SEQ ID NO: 150 (VB4B: VH),
- (16) SEQ ID NO: 152 (VB51: VH),
- 5 (17) SEQ ID NO: 155 (AB317: VH),
- (18) SEQ ID NO: 159 (AB324: VH),
- (19) SEQ ID NO: 162 (TA136: VH).

[0028]

(IV)

10 Antibody comprising a VL that comprises an amino acid sequence of the SEQ ID NO in any one of (1) to (19):

- (1) SEQ ID NO: 125 (VA7: VL),
- (2) SEQ ID NO: 127 (VA130: VL),
- (3) SEQ ID NO: 129 (VA259: VL),
- 15 (4) SEQ ID NO: 131 (VB17B: VL),
- (5) SEQ ID NO: 133 (VB12B: VL),
- (6) SEQ ID NO: 135 (VB140: VL),
- (7) SEQ ID NO: 137 (VB33: VL),
- (8) SEQ ID NO: 139 (VB45B: VL),
- 20 (9) SEQ ID NO: 141 (VB8B: VL),
- (10) SEQ ID NO: 143 (VB115: VL),
- (11) SEQ ID NO: 145 (VB14B: VL),
- (12) SEQ ID NO: 120 (VB22B: VL),
- (13) SEQ ID NO: 147 (VB16: VL),
- 25 (14) SEQ ID NO: 149 (VB157: VL),
- (15) SEQ ID NO: 151 (VB4B: VL),
- (16) SEQ ID NO: 153 (VB51: VL),
- (17) SEQ ID NO: 157 (AB317: VL),
- (18) SEQ ID NO: 161 (AB324: VL),
- 30 (19) SEQ ID NO: 163 (TA136: VL).

[0029]

(V)

Antibody comprising a VH and VL according to any one of (1) to (19):

- (1) SEQ ID NOs: 3, 4, and 5 (VA7: H chain CDR1, 2, and 3); SEQ ID NOs: 60, 61, and 62
- 35 (VA7: L chain CDR1, 2, and 3),
- (2) SEQ ID NOs: 6, 7, and 8 (VA130: H chain CDR1, 2, and 3), SEQ ID NOs: 63, 64, and 65

- (VA130: L chain CDR1, 2, and 3),
 (3) SEQ ID NOs: 9, 10, and 11 (VA259: H chain CDR1, 2, and 3); SEQ ID NOs: 66, 67, and 68
 (VA259: L chain CDR1, 2, and 3),
 (4) SEQ ID NOs: 12, 13, and 14 (VB17B: H chain CDR1, 2, and 3); SEQ ID NOs: 69, 70, and
 5 71 (VB17B: L chain CDR1, 2, and 3),
 (5) SEQ ID NOs: 15, 16, and 17 (VB12B: H chain CDR1, 2, and 3); SEQ ID NOs: 72, 73, and
 74 (VB12B: L chain CDR1, 2, and 3),
 (6) SEQ ID NOs: 18, 19, and 20 (VB140: H chain CDR1, 2, and 3); SEQ ID NOs: 75, 76, and 77
 (VB140: L chain CDR1, 2, and 3),
 10 (7) SEQ ID NOs: 21, 22, and 23 (VB33: H chain CDR1, 2, and 3); SEQ ID NOs: 78, 79, and 80
 (VB33: L chain CDR1, 2, and 3),
 (8) SEQ ID NOs: 24, 25, and 26 (VB45B: H chain CDR1, 2, and 3); SEQ ID NOs: 81, 82, and
 83 (VB45B: L chain CDR1, 2, and 3),
 (9) SEQ ID NOs: 27, 28, and 29 (V8B: H chain CDR1, 2, and 3); SEQ ID NOs: 84, 85, and 86
 15 (V8B: L chain CDR1, 2, and 3),
 (10) SEQ ID NOs: 30, 31, and 32 (VB115: H chain CDR1, 2, and 3); SEQ ID NOs: 87, 88, and
 89 (VB115: L chain CDR1, 2, and 3),
 (11) SEQ ID NOs: 33, 34, and 35 (V14B: H chain CDR1, 2, and 3); SEQ ID NOs: 90, 91, and 92
 (VB14B: L chain CDR1, 2, and 3),
 20 (12) SEQ ID NOs: 36, 37, and 38 (V22B: H chain CDR1, 2, and 3); SEQ ID NOs: 93, 94, and 95
 (VB22B: L chain CDR1, 2, and 3),
 (13) SEQ ID NOs: 39, 40, and 41 (VB16: H chain CDR1, 2, and 3); SEQ ID NOs: 96, 97, and 98
 (VB16: L chain CDR1, 2, and 3),
 (14) SEQ ID NOs: 42, 43, and 44 (VB157: H chain CDR1, 2, and 3); SEQ ID NOs: 99, 100, and
 25 101 (VB157: L chain CDR1, 2, and 3),
 (15) SEQ ID NOs: 45, 46, and 47 (VB4B: H chain CDR1, 2, and 3); SEQ ID NOs: 102, 103, and
 104 (VB4B: L chain CDR1, 2, and 3),
 (16) SEQ ID NOs: 48, 49, and 50 (VB51: H chain CDR1, 2, and 3); SEQ ID NOs: 105, 106, and
 107 (VB51: L chain CDR1, 2, and 3),
 30 (17) SEQ ID NOs: 51, 52, and 53 (AB317: H chain CDR1, 2, and 3); SEQ ID NOs: 108, 109,
 and 110 (AB317: L chain CDR1, 2, and 3),
 (18) SEQ ID NOs: 54, 55, and 56 (AB324: H chain CDR1, 2, and 3); SEQ ID NOs: 111, 112,
 and 113 (AB324: L chain CDR1, 2, and 3),
 (19) SEQ ID NOs: 57, 58, and 59 (TA136: H chain CDR1, 2, and 3); SEQ ID NOs: 114, 115, and
 35 116 (TA136: L chain CDR1, 2, and 3).

[0030]

(VI)

Antibody comprising a VH and a VL that comprise the amino acid sequences according to SEQ ID NOs in any one of (1) to (19) indicated below:

- (1) SEQ ID NO: 124 (VA7: VH), SEQ ID NO: 125 (VA7: VL),
- 5 (2) SEQ ID NO: 126 (VA130: VH), SEQ ID NO: 127 (VA130: VL),
- (3) SEQ ID NO: 128 (VA259: VH), SEQ ID NO: 129 (VA259: VL),
- (4) SEQ ID NO: 130 (VB17B: VH), SEQ ID NO: 131 (VB17B: VL),
- (5) SEQ ID NO: 132 (VB12B: VH), SEQ ID NO: 133 (VB12B: VL),
- (6) SEQ ID NO: 134 (VB140: VH), SEQ ID NO: 135 (VB140: VL),
- 10 (7) SEQ ID NO: 136 (VB33: VH), SEQ ID NO: 137 (VB33: VL),
- (8) SEQ ID NO: 138 (VB45B: VH), SEQ ID NO: 139 (VB45B: VL),
- (9) SEQ ID NO: 140 (V8B: VH), SEQ ID NO: 141 (VB8B: VL),
- (10) SEQ ID NO: 142 (VB115: VH), SEQ ID NO: 143 (VB115: VL),
- (11) SEQ ID NO: 144 (VB14B: VH), SEQ ID NO: 145 (VB14B: VL),
- 15 (12) SEQ ID NO: 118 (VB22B: VH), SEQ ID NO: 120 (VB22B: VL),
- (13) SEQ ID NO: 146 (VB16: VH), SEQ ID NO: 147 (VB16: VL),
- (14) SEQ ID NO: 148 (VB157: VH), SEQ ID NO: 149 (VB157: VL),
- (15) SEQ ID NO: 150 (VB4B: VH), SEQ ID NO: 151 (VB4B: VL),
- (16) SEQ ID NO: 152 (VB51: VH), SEQ ID NO: 153 (VB51: VL),
- 20 (17) SEQ ID NO: 155 (AB317: VH), SEQ ID NO: 157 (AB317: VL),
- (18) SEQ ID NO: 159 (AB324: VH), SEQ ID NO: 161 (AB324: VL),
- (19) SEQ ID NO: 162 (TA136: VH), SEQ ID NO: 163 (TA136: VL).

[0031]

(VII)

- 25 Antibody comprising the amino acid sequence of SEQ ID NO: 122 (VB22B: scFv).

[0032]

(VIII)

Humanized antibody comprising an amino acid sequence according to any one of SEQ ID NO: 2 (VB22B: sc(Fv)₂).

- 30 [0033]

(IX)

An antibody comprising an amino acid sequence of any one of (I) to (VIII) indicated above, in which one or more amino acids have been substituted, deleted, added, and/or inserted, wherein the antibody has activity equivalent to that of the antibody of any one of (I) to (V).

- 35 [0034]

Herein, the phrase “functionally equivalent” means that an antibody of interest has a

biological or biochemical activity comparable to that of an antibody of the present invention. Such activities include, for example, binding activities and agonistic activities.

[0035]

5 Methods for preparing polypeptides functionally equivalent to a certain polypeptide are well known to those skilled in the art, and include methods of introducing mutations into polypeptides. For example, those skilled in the art can prepare an antibody functionally equivalent to the antibodies of the present invention by introducing appropriate mutations into the antibody using site-directed mutagenesis (Hashimoto-Gotoh, T. *et al.* Gene (1995) 152: 271-275; Zoller, MJ, and Smith, M. Methods Enzymol. (1983) 100: 468-500; Kramer, W. *et al.*,
10 Nucleic Acids Res. (1984) 12: 9441-9456; Kramer, W. and Fritz HJ, Methods Enzymol. (1987) 154: 350-367; Kunkel, TA, Proc. Natl. Acad. Sci. USA (1985) 82: 488-492; Kunkel, Methods Enzymol. (1988) 85: 2763-2766), or such. Amino acid mutations may occur naturally. Thus, the present invention also comprises antibodies functionally equivalent to the antibodies of the present invention and comprising the amino acid sequences of these antibodies, in which one or
15 more amino acids is mutated. Generally, the number of amino acids that are mutated is 50 amino acids or less, preferably 30 or less, more preferably 10 or less (for example, five amino acids or less).

[0036]

20 An amino acid is preferably substituted for a different amino acid(s) that allows the properties of the amino acid side-chain to be conserved. Examples of amino acid side chain properties are: hydrophobic amino acids (A, I, L, M, F, P, W, Y, and V), hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, and T), amino acids comprising the following side chains: aliphatic side chains (G, A, V, L, I, and P); hydroxyl-containing side chains (S, T, and Y); sulfur-containing side chains (C and M); carboxylic acid- and amide-containing side chains (D,
25 N, E, and Q); basic side chains (R, K, and H); aromatic ring-containing side chains (H, F, Y, and W) (amino acids are represented by one-letter codes in parentheses).

[0037]

A polypeptide comprising a modified amino acid sequence, in which one or more amino acid residues is deleted, added, and/or replaced with other amino acids, is known to retain its
30 original biological activity (Mark, D. F. *et al.*, Proc. Natl. Acad. Sci. USA (1984) 81: 5662-5666; Zoller, M. J. & Smith, M. Nucleic Acids Research (1982) 10: 6487-6500; Wang, A. *et al.*, Science 224, 1431-1433; Dalbadie-McFarland, G. *et al.*, Proc. Natl. Acad. Sci. USA (1982) 79: 6409-6413).

[0038]

35 Fusion proteins containing antibodies that comprise the amino acid sequence of an antibody of the present invention, in which two or more amino acid residues have been added,

are included in the present invention. The fusion protein results from a fusion between one of the above antibodies and a second peptide or protein, and is included in the present invention. The fusion protein can be prepared by ligating a polynucleotide encoding an antibody of the present invention and a polynucleotide encoding a second peptide or polypeptide in frame, inserting this into an expression vector, and expressing the fusion construct in a host. Some techniques known to those skilled in the art are available for this purpose. The partner peptide or polypeptide to be fused with an antibody of the present invention may be a known peptide, for example, FLAG (Hopp, T. P. *et al.*, BioTechnology (1988) 6: 1204-1210), 6x His consisting of six His (histidine) residues, 10x His, influenza hemagglutinin (HA), human c-myc fragment, VSV-GP fragment, p18HIV fragment, T7-tag, HSV-tag, E-tag, SV40 T antigen fragment, Ick tag, α -tubulin fragment, B-tag, Protein C fragment. Other partner polypeptides to be fused with the antibodies of the present invention include, for example, GST (glutathione-S-transferase), HA (influenza hemagglutinin), immunoglobulin constant region, β -galactosidase, and MBP (maltose-binding protein). A polynucleotide encoding one of these commercially available peptides or polypeptides can be fused with a polynucleotide encoding an antibody of the present invention. The fusion polypeptide can be prepared by expressing the fusion construct.

[0039]

As described below, the antibodies of the present invention may differ in amino acid sequence, molecular weight, isoelectric point, presence/absence of sugar chains, and conformation depending on the cell or host producing the antibody, or purification method. However, a resulting antibody is included in the present invention, as long as it is functionally equivalent to an antibody of the present invention. For example, when an antibody of the present invention is expressed in prokaryotic cells, for example *E. coli*, a methionine residue is added to the N terminus of the original antibody amino acid sequence. Such antibodies are included in the present invention.

[0040]

An antibody that recognizes an epitope recognized by the antibody according to any one of (I) to (IX) indicated above is expected to have a high agonistic activity. Such antibodies can be prepared by methods known to those skilled in the art. The antibody can be prepared by, for example, determining the epitope recognized by the antibody according to any one of (I) to (IX) by conventional methods, and using a polypeptide comprising one of the epitope amino acid sequences as an immunogen. Alternatively, the antibody can be prepared by determining the epitopes of conventionally prepared antibodies and selecting an antibody that recognizes the epitope recognized by an antibody of any one of (I) to (IX).

[0041]

In the present invention, a particularly preferred antibody is an antibody that recognizes

the epitope recognized by the antibody comprising the amino acid sequence of SEQ ID NO: 2. The antibody comprising the amino acid sequence of SEQ ID NO: 2 is predicted to recognize the region from Glu 26 to Leu 274, preferably the region from Ala 189 to Gly 245, more preferably the region from Gln 213 to Ala 231 of human Mpl. Thus, antibodies recognizing the region of amino acids 26 to 274, or amino acids 189 to 245, or amino acids 213 to 231 of human Mpl are also included in the present invention.

[0042]

Antibodies recognizing regions of amino acids 26 to 274, amino acids 189 to 245, or amino acids 213 to 231 of the human Mpl amino acid sequence (SEQ ID NO: 123) can be obtained by methods known to those skilled in the art. Such antibodies can be prepared by, for example, using a peptide comprising amino acids 26 to 274, amino acids 189 to 245, or amino acids 213 to 231 of the human Mpl amino acid sequence (SEQ ID NO: 123) as an immunogen. Alternatively, such antibodies can be prepared by determining the epitope of a conventionally prepared antibody and selecting an antibody that recognizes the same epitope recognized by an antibody of the present invention.

[0043]

The present invention provides antibodies described above in (I) to (IX). In an embodiment of the present invention, a preferred antibody is the one shown in (V), a more preferred antibody is the one shown in (VI), and a still more preferred is the one shown in (VII) or (VIII).

[0044]

The present invention also provides vectors comprising polynucleotides encoding the antibodies of the present invention, or polynucleotides which hybridize under stringent conditions to the polynucleotides of the present invention and encode antibodies having activities equivalent to those of the antibodies of the present invention. The polynucleotides of the present invention are polymers comprising multiple bases or base pairs of deoxyribonucleic acids (DNA) or ribonucleic acids (RNA), and are not particularly limited, as long as they encode the antibodies of the present invention. They may also contain non-natural nucleotides. The polynucleotides of the present invention can be used to express antibodies using genetic engineering techniques. The polynucleotides of this invention can also be used as probes in the screening of antibodies functionally equivalent to the antibodies of the present invention. Specifically, DNAs that hybridize under stringent conditions to a polynucleotide encoding an antibody of the present invention, and encode antibodies having activity equivalent to those of the antibodies of the present invention can be obtained by techniques such as hybridization and gene amplification (for example, PCR), using a polynucleotide of the present invention or a portion thereof as a probe. Such DNAs are also included in the polynucleotides of the present

invention. Hybridization techniques are well known to those skilled in the art (Sambrook, J *et al.*, Molecular Cloning 2nd ed., 9.47-9.58, Cold Spring Harbor Lab. press, 1989). Such hybridization conditions include, for example, conditions of low stringency. Examples of conditions of low stringency include post-hybridization washing in 0.1x SSC and 0.1% SDS at 42°C, and preferably in 0.1x SSC and 0.1% SDS at 50°C. More preferable hybridization conditions include those of high stringency. Highly stringent conditions include, for example, washing in 5x SSC and 0.1% SDS at 65°C. In these conditions, the higher the temperature, the higher the expectation of efficiently obtaining polynucleotides with a high homology. However, several factors, such as temperature and salt concentration, can influence hybridization stringency, and those skilled in the art can suitably select these factors to accomplish similar stringencies.

[0045]

Antibodies that are encoded by polynucleotides obtained by the hybridization and gene amplification techniques, and are functionally equivalent to the antibodies of the present invention generally exhibit high homology to the antibodies of the this invention at the amino acid level. The antibodies of the present invention include antibodies that are functionally equivalent to the antibodies of the present invention, and exhibit high amino acid sequence homology to the antibodies of this invention. The term "high homology" generally means identity at the amino acid level of at least 50% or higher, preferably 75% or higher, more preferably 85% or higher, still more preferably 95% or higher. Polypeptide homology can be determined by the algorithm described in the report: Wilbur, W. J. and Lipman, D. J. Proc. Natl. Acad. Sci. USA (1983) 80: 726-730.

[0046]

When *E. coli* is used as a host, there is no particular limitation as to the type of vector of the present invention, as long as the vector contains an "ori" responsible for its replication in *E. coli* and a marker gene. The "ori" ensures the amplification and mass production of the vector in *E. coli* (for example, JM109, DH5α, HB101, and XL1Blue). The marker gene is used to select the *E. coli* transformants (for example, a drug resistance gene selected by an appropriate drug such as ampicillin, tetracycline, kanamycin, and chloramphenicol). The vectors include, for example, M13 vectors, pUC vectors, pBR322, pBluescript, and pCR-Script. In addition to the above vectors, for example, pGEM-T, pDIRECT, and pT7 can also be used for the subcloning and excision of cDNAs.

[0047]

An expression vector is especially useful for the type of vectors of the present invention. When an expression vector is expressed, for example, in *E. coli*, it should have the above characteristics in order to be amplified in *E. coli*. Additionally, when *E. coli*, such as JM109,

DH5 α , HB101, or XL1-Blue are used as the host cell, the vector preferably has a promoter, for example, lacZ promoter (Ward *et al.* Nature (1989) 341: 544-546; FASEB J. (1992) 6: 2422-2427), araB promoter (Better *et al.*, Science (1988) 240: 1041-1043), or T7 promoter, that allows efficient expression of the desired gene in *E. coli*. Other examples of the vectors include
5 pGEX-5X-1 (Pharmacia), "QIAexpress system" (QIAGEN), pEGFP, and pET (where BL21, a strain expressing T7 RNA polymerase, is preferably used as the host).

[0048]

Furthermore, the vector may comprise a signal sequence for polypeptide secretion. When producing polypeptides into the periplasm of *E. coli*, the pelB signal sequence (Lei, S. P.
10 *et al.* J. Bacteriol. (1987) 169: 4379) may be used as a signal sequence for polypeptide secretion. For example, calcium chloride methods or electroporation methods may be used to introduce the vector into a host cell.

[0049]

In addition to *E. coli*, expression vectors derived from mammals (e.g., pCDNA3
15 (Invitrogen), pEGF-BOS (Nucleic Acids Res. (1990) 18 (17): 5322), pEF, pCDM8), insect cells (e.g., "Bac-to-BAC baculovirus expression system" (GIBCO-BRL), pBacPAK8), plants (e.g., pMH1, pMH2), animal viruses (e.g., pHSV, pMV, pAdexLcw), retroviruses (e.g., pZIPneo), yeasts (e.g., "Pichia Expression Kit" (Invitrogen), pNV11, SP-Q01), and *Bacillus subtilis* (e.g., pPL608, pKTH50) may also be used as a vector of the present invention.

20 [0050]

In order to express proteins in animal cells such as CHO, COS, and NIH3T3 cells, the vector preferably has a promoter necessary for expression in such cells, for example, an SV40 promoter (Mulligan *et al.* Nature (1979) 277: 108), MMLV-LTR promoter, EF1 α promoter (Mizushima *et al.* Nucleic Acids Res. (1990) 18: 5322), CMV promoter, etc.). It is even more
25 preferable that the vector also carries a marker gene for selecting transformants (for example, a drug-resistance gene selected by a drug such as neomycin and G418). Examples of vectors with such characteristics include pMAM, pDR2, pBK-RSV, pBK-CMV, pOPRSV, and pOP13, and such.

[0051]

30 In addition, to stably express a gene and amplify the gene copy number in cells, CHO cells that are defective in the nucleic acid synthesis pathway are introduced with a vector containing a DHFR gene (for example, pCHOI) to compensate for the defect, and the copy number is amplified using methotrexate (MTX). Alternatively, a COS cell, which carries an SV40 T antigen-expressing gene on its chromosome, can be transformed with a vector
35 containing the SV40 replication origin (for example, pcD) for transient gene expression. The replication origin may be derived from polyoma virus, adenovirus, bovine papilloma virus

(BPV), and such. Furthermore, to increase the gene copy number in host cells, the expression vector may contain, as a selection marker, aminoglycoside transferase (APH) gene, thymidine kinase (TK) gene, *E. coli* xanthine guanine phosphoribosyl transferase (Ecogpt) gene, dihydrofolate reductase (dhfr) gene, and such.

5 [0052]

Next, the vector is introduced into a host cell. The host cells into which the vector is introduced are not particularly limited, for example, *E. coli* and various animal cells are available for this purpose. The host cells may be used, for example, as a production system to produce and express the antibodies of the present invention. *In vitro* and *in vivo* production systems are
10 available for polypeptide production systems. Production systems that use eukaryotic cells or prokaryotic cells are examples of *in vitro* production systems.

[0053]

Eukaryotic cells that can be used are, for example, animal cells, plant cells, and fungal cells. Known animal cells include: mammalian cells, for example, CHO (J. Exp. Med. (1995)
15 108: 945), COS, 3T3, myeloma, BHK (baby hamster kidney), HeLa, Vero, amphibian cells such as *Xenopus laevis* oocytes (Valle, *et al.*, Nature (1981) 291: 358-340), or insect cells (e.g., Sf9, Sf21, and Tn5). In the present invention, CHO-DG44, CHO-DXB11, COS7 cells, and BHK cells can be suitably used. Among animal cells, CHO cells are particularly favorable for large-scale expression. Vectors can be introduced into a host cell by, for example, calcium
20 phosphate methods, the DEAE-dextran methods, methods using cationic liposome DOTAP (Boehringer-Mannheim), electroporation methods, lipofection methods.

[0054]

Plant cells include, for example, *Nicotiana tabacum*-derived cells known as a protein production system. Calluses may be cultured from these cells. Known fungal cells include
25 yeast cells, for example, genus *Saccharomyces* such as *Saccharomyces cerevisiae* and *Saccharomyces pombe*; and filamentous fungi, for example, genus *Aspergillus* such as *Aspergillus niger*.

[0055]

Bacterial cells can be used in the prokaryotic production systems. Examples of
30 bacterial cells include *E. coli* (for example, JM109, DH5 α , HB101 and such); and *Bacillus subtilis*.

[0056]

Next, the above host cells are cultured. Antibodies can be obtained by transforming the cells with a polynucleotide of interest and *in vitro* culturing of these transformants.
35 Transformants can be cultured using known methods. For example, DMEM, MEM, RPMI 1640, or IMDM may be used as the culture medium for animal cells, and may be used with or

without serum supplements such as FBS or fetal calf serum (FCS). Serum-free cultures are also acceptable. The preferred pH is about 6 to 8 during the course of culturing. Incubation is carried out typically at a temperature of about 30 to 40°C for about 15 to 200 hours. Medium is exchanged, aerated, or agitated, as necessary.

5 [0057]

On the other hand, production systems using animal or plant hosts may be used as systems for producing polypeptides *in vivo*. For example, a polynucleotide of interest is introduced into an animal or plant and the polypeptide is produced in the body of the animal or plant and then recovered. The "hosts" of the present invention includes such animals and
10 plants.

[0058]

Animals to be used for the production system include mammals or insects. Mammals such as goats, pigs, sheep, mice, and cattle may be used (Vicki Glaser SPECTRUM Biotechnology Applications (1993)). Alternatively, the mammals may be transgenic animals.

15 For example, a polynucleotide of interest is prepared as a fusion gene with a gene encoding a polypeptide specifically produced in milk, such as the goat β -casein gene. DNA fragments containing the fusion gene are injected into goat embryos, which are then introduced back to female goats. The desired antibody can be obtained from milk produced by the transgenic goats, which are born from the goats that received the embryos, or from their
20 offspring. Appropriate hormones may be administered to increase the volume of milk containing the antibody produced by the transgenic goats (Ebert, K.M. *et al.*, Bio/Technology (1994) 12: 699-702).

[0059]

Insects, such as silkworms, may also be used. Baculoviruses carrying a polynucleotide
25 encoding an antibody of interest can be used to infect silkworms, and the antibody of interest can be obtained from the body fluids (Susumu, M. *et al.*, Nature (1985) 315: 592-594).

[0060]

Plants used in the production system include, for example, tobacco. When tobacco is used, a polynucleotide encoding an antibody of interest is inserted into a plant expression vector,
30 for example, pMON 530, and then the vector is introduced into a bacterium, such as *Agrobacterium tumefaciens*. The bacteria are then used to infect tobacco such as *Nicotiana tabacum*, and the desired antibodies can be recovered from the leaves (Julian K.-C. Ma *et al.*, Eur. J. Immunol. (1994) 24: 131-138).

[0061]

35 The resulting antibody may be isolated from the inside or outside (such as the medium) of host cells, and purified as a substantially pure and homogenous antibody. Methods are not

limited to any specific method and any standard method for isolating and purifying antibodies may be used. Polypeptides may be isolated and purified, by selecting an appropriate combination of, for example, chromatographic columns, filtration, ultrafiltration, salting out, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, isoelectric focusing, dialysis, recrystallization, and others.

Chromatographies include, for example, affinity chromatographies, ion exchange chromatographies, hydrophobic chromatographies, gel filtrations, reverse-phase chromatographies, and adsorption chromatographies (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak *et al.*, Cold Spring Harbor Laboratory Press, 1996). These chromatographies can be carried out using liquid phase chromatographies such as HPLC and FPLC. Examples of the affinity chromatography columns include protein A columns and protein G columns. Examples of the proteins A columns include Hyper D, POROS, and Sepharose F. F. (Pharmacia).

[0062]

An antibody can be modified freely and peptide portions deleted by treating the antibody with an appropriate protein modifying enzyme before or after antibody purification. Such protein modifying enzymes include, for example, trypsins, chymotrypsins, lysyl endopeptidases, protein kinases, and glucosidases.

[0063]

Antibodies that bind to Mpl can be prepared by methods known to those skilled in the art.

For example, monoclonal antibody-producing hybridomas can be essentially generated by known technologies as follows: immunizing animals with Mpl proteins or Mpl-expressing cells as sensitized antigens using conventional immunological methods; fusing the obtained immunocytes with known parental cells by conventional cell fusion methods; and screening for monoclonal antibody-producing cells by conventional methods.

Specifically, monoclonal antibodies can be prepared by the method below.

First, Mpl protein, which is used as a sensitized antigen for preparing antibodies, is prepared by expressing the Mpl gene/amino acid sequence (GenBank accession number: NM_005373). More specifically, the gene sequence encoding Mpl is inserted into a known expression vector, which is then transfected into an appropriate host cell. The subject human Mpl protein is purified from the host cell or culture supernatant using known methods.

The purified Mpl protein is then used as a sensitized antigen. Alternatively, a partial Mpl peptide may be used as a sensitized antigen. In this case, the partial peptide can also be chemically synthesized based on the amino acid sequence of human Mpl.

[0064]

The epitopes of Mpl molecule that are recognized by an anti-Mpl antibody of the present invention are not limited to a particular epitope, and may be any epitope on the Mpl molecule. Thus, any fragment can be used as an antigen for preparing anti-Mpl antibodies of the present invention, as long as the fragment comprises an epitope of the Mpl molecule.

5 [0065]

There is no limitation as to the type of mammalian species to be immunized with the sensitized antigen. However, a mammal is preferably selected based on its compatibility with the parental cell to be used in cell fusion. Generally, rodents (for example, mice, rats, and hamsters), rabbits, and monkeys can be used.

10 [0066]

Animals can be immunized with a sensitized antigen by known methods such as a routine method of injecting a sensitized antigen into a mammal intraperitoneally or subcutaneously. Specifically, the sensitized antigen is diluted appropriately with phosphate-buffered saline (PBS), physiological saline and such, and then suspended. An adequate amount of a conventional adjuvant, for example, Freund's complete adjuvant, is mixed with the suspension, as necessary. An emulsion is then prepared for administering to a mammal several times over a 4- to 21-day interval. An appropriate carrier may be used for the sensitized antigen in immunization.

A mammal is immunized as described above. After a titer increase of target antibody in the serum is confirmed, immunocytes are collected from the mammal and then subjected to cell fusion. Spleen cells are the preferred immunocytes.

[0067]

Mammalian myeloma cells are used as the parental cells to be fused with the above immunocytes. Preferable myeloma cells to be used include various known cell lines, for example, P3 (P3x63Ag8.653) (Kearney JF, *et al.*, J. Immunol. (1979) 123: 1548-1550), P3x63Ag8U.1 (Yelton DE, *et al.*, Current Topics in Microbiology and Immunology (1978) 81: 1-7), NS-1 (Kohler, G. and Milstein, C. Eur. J. Immunol. (1976) 6: 511-519), MPC-11 (Margulies, D. H. *et al.*, Cell (1976) 8: 405-415), SP2/0 (Shulman, M. *et al.*, Nature (1978) 276: 269-270), FO (deSt. Groth, S. F. *et al.*, J. Immunol. Methods (1980) 35: 1-21), S194 (Trowbridge, I. S., J. Exp. Med. (1978) 148: 313-323), and R210 (Galfre, G. *et al.*, Nature (1979) 277: 131-133).

[0068]

Cell fusions between the immunocytes and the myeloma cells as described above can be essentially carried out using known methods, for example, a method by Kohler and Milstein (Kohler, G. and Milstein, C., Methods Enzymol. (1981) 73: 3-46).

35 [0069]

More specifically, the above-described cell fusions are carried out, for example, in a

conventional culture medium in the presence of a cell fusion-promoting agent. The fusion-promoting agents include, for example, polyethylene glycol (PEG) and Sendai virus (HVJ). If required, an auxiliary substance such as dimethyl sulfoxide may also be added to improve fusion efficiency.

5 [0070]

The ratio of immunocytes to myeloma cells may be determined at one's own discretion, preferably, for example, one myeloma cell for every one to ten immunocytes. Culture media to be used for the above cell fusions include, for example, media that are suitable for the growth of the above myeloma cell lines, such as RPMI 1640 media and MEM media, and other
10 conventional culture media used for this type of cell culture. In addition, serum supplements such as fetal calf serum (FCS) may also be used in combination.

[0071]

Cell fusion is carried out as follows. As described above, predetermined amounts of immunocytes and myeloma cells are mixed well in the culture medium. PEG solution (for
15 example, mean molecular weight of about 1,000-6,000) pre-heated to 37°C is added to the cell suspension typically at a concentration of 30% to 60% (w/v), and mixed to produce fused cells (hybridomas). Then, an appropriate culture medium is successively added to the mixture, and the sample is centrifuged to remove supernatant. This treatment is repeated several times to remove the unwanted cell fusion-promoting agent and others that are unfavorable to hybridoma
20 growth.

[0072]

Screening of the resulting hybridomas can be carried out by culturing them in a conventional selective medium, for example, hypoxanthine, aminopterin, and thymidine (HAT) medium. Culturing in the above-described HAT medium is continued for a period long enough
25 (typically, for several days to several weeks) to kill cells (non-fused cells) other than the desired hybridomas. Then, hybridomas are screened for single-cell clones capable of producing the target antibody by conventional limiting dilution methods.

[0073]

In addition to the method for preparing the above-described hybridomas by immunizing
30 non-human animals with antigens, preferred human antibodies having binding activity to Mpl can also be obtained by: sensitizing human lymphocytes with Mpl *in vitro*; and fusing the sensitized lymphocytes with human myeloma cells capable of dividing permanently (see, Japanese Patent Application Kokoku Publication No. (JP-B) H01-59878 (examined, approved Japanese patent application published for opposition)). Alternatively, it is possible to obtain
35 human antibodies against Mpl from immortalized cells producing anti-Mpl antibodies. In this method, the cells producing anti-Mpl antibodies are prepared by administering Mpl as an antigen

to transgenic animals comprising a repertoire of the entire human antibody genes (see, WO 94/25585, WO 93/12227, WO 92/03918, and WO 94/02602).

The monoclonal antibody-producing hybridomas thus prepared can be passaged in a conventional culture medium, and stored in liquid nitrogen over long periods of time.

[0074]

Monoclonal antibodies can be prepared from the above-described hybridomas by, for example, a routine procedure of culturing the hybridomas and obtaining antibodies from the culture supernatants. Alternatively, monoclonal antibodies can be prepared by injecting the hybridomas into a compatible mammal; growing these hybridomas in the mammal; and obtaining antibodies from the mammal's ascites. The former method is suitable for preparing highly purified antibodies, while the latter is suitable for preparing antibodies on a large scale.

[0075]

Recombinant antibodies can also be prepared by: cloning an antibody gene from a hybridoma; inserting the gene into an appropriate vector; introducing the vector into a host; and producing the antibodies by using genetic recombination techniques (see, for example, Vandamme, A. M. *et al.*, Eur. J. Biochem. (1990) 192: 767-775).

[0076]

Specifically, an mRNA encoding the variable (V) region of anti-Mpl antibody is isolated from hybridomas producing the anti-Mpl antibodies. For mRNA isolation, total RNAs are first prepared by conventional methods such as guanidine ultracentrifugation methods (Chirgwin, J. M. *et al.*, Biochemistry (1979) 18: 5294-5299), or acid guanidinium thiocyanate-phenol-chloroform (AGPC) methods (Chomczynski, P. *et al.*, Anal. Biochem. (1987) 162: 156-159), and then the target mRNA is prepared using an mRNA Purification Kit (Pharmacia) and such. Alternatively, the mRNA can be directly prepared using the QuickPrep mRNA Purification Kit (Pharmacia).

[0077]

A cDNA of the antibody V region is synthesized from the resulting mRNA using reverse transcriptase. cDNA synthesis is carried out using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Co.), or such. Alternatively, cDNA can be synthesized and amplified by the 5'-RACE method (Frohman, M. A. *et al.*, Proc. Natl. Acad. Sci. USA (1988) 85: 8998-9002; Belyavsky, A. *et al.*, Nucleic Acids Res. (1989) 17: 2919-2932) using the 5'-Ampli FINDER RACE Kit (Clontech) and PCR.

[0078]

Target DNA fragments are purified from the obtained PCR products and then ligated with vector DNAs to prepare recombinant vectors. The vectors are introduced into *E. coli* and such, and colonies are selected for preparing the recombinant vector of interest. The target

DNA nucleotide sequence is then confirmed by conventional methods such as the dideoxynucleotide chain termination method.

Once a DNA encoding the V region of target anti-Mpl antibody is obtained, the DNA is inserted into an expression vector which comprises a DNA encoding the constant region (C region) of a desired antibody.

The method for producing anti-Mpl antibodies to be used in the present invention typically comprises the steps of: inserting an antibody gene into an expression vector, so that the gene is expressed under the regulation of expression regulatory regions, such as enhancer and promotor; and transforming host cells with the resulting vectors to express antibodies.

For expressing the antibody gene, polynucleotides encoding H chain and L chain, respectively, are inserted into separate expression vectors and co-transfected into a host cell. Alternatively, polynucleotides encoding both H chain and L chain are inserted into a single expression vector and transfected into a host cell (see WO 94/11523).

[0079]

The term “agonistic activity” refers to an activity to induce changes in some biological activities through signal transduction into cells and such, due to the binding of an antibody to a receptor antigen. The biological activities include, for example, proliferation-promoting activities, proliferation activities, viability activities, differentiation-inducing activities, differentiation activities, transcriptional activities, membrane transport activities, binding activities, proteolytic activities, phosphorylation/dephosphorylation activities, oxidation/reduction activities, transfer activities, nucleolytic activities, dehydration activities, cell death-inducing activities, and apoptosis-inducing activities, but is not limited thereto.

The term “agonistic activity against Mpl” typically refers to the activity of promoting the differentiation of megakaryocytes or their parental hemopoietic stem cells into platelets, or the activity of stimulating platelet proliferation.

Agonistic activity can be assayed by methods known to those skilled in the art. The agonistic activity may be determined using the original activity or a different activity as an indicator.

For example, agonistic activity can be determined by a method using cell growth as an indicator as described in Examples. More specifically, an antibody whose agonistic activity is to be determined is added to cells which proliferate in an agonist-dependent manner, followed by incubation of the cells. Then, a reagent such as WST-8, which shows a coloring reaction at specific wavelengths depending on the viable cell count, is added to the culture and absorbance is measured. The agonistic activity can be determined using the measured absorbance as an indicator.

[0080]

Cells that proliferate in an agonist-dependent manner can also be prepared by methods known to those skilled in the art. For example, when the antigen is a receptor capable of transducing cell growth signals, cells expressing the receptor may be used. Alternatively, when the antigen is a receptor that cannot transduce signals, a chimeric receptor consisting of the intracellular domain of a receptor that transduces cell growth signals and the extracellular domain of a receptor that does not transduce cell growth signals can be prepared for cellular expression. Receptors that transduce cell growth signals include, for example, G-CSF receptors, mpl, neu, GM-CSF receptors, EPO receptors, c-kit, and FLT-3. Cells that can be used to express a receptor include, for example, BaF3, NFS60, FDCP-1, FDCP-2, CTLL-2, DA-1, and KT-3.

[0081]

There is no limitation as to the type of detection indicators to be used for determining agonistic activity, as long as the indicator can monitor quantitative and/or qualitative changes. For example, it is possible to use cell-free assay indicators, cell-based assay indicators, tissue-based assay indicators, and *in vivo* assay indicators. Indicators that can be used in cell-free assays include enzymatic reactions, quantitative and/or qualitative changes in proteins, DNAs, or RNAs. Such enzymatic reactions include, for example, amino acid transfers, sugar transfers, dehydrations, dehydrogenations, and substrate cleavages. Alternatively, protein phosphorylations, dephosphorylations, dimerizations, multimerizations, hydrolyses, dissociations and such; DNA or RNA amplifications, cleavages, and extensions can be used as the indicator in cell-free assays. For example, protein phosphorylations downstream of a signal transduction pathway may be used as a detection indicator. Alterations in cell phenotype, for example, quantitative and/or qualitative alterations in products, alterations in growth activity, alterations in cell number, morphological alterations, or alterations in cellular properties, can be used as the indicator in cell-based assays. The products include, for example, secretory proteins, surface antigens, intracellular proteins, and mRNAs. The morphological alterations include, for example, alterations in dendrite formation and/or dendrite number, alteration in cell flatness, alteration in cell elongation/axial ratio, alterations in cell size, alterations in intracellular structure, heterogeneity/homogeneity of cell populations, and alterations in cell density. Such morphological alterations can be observed under a microscope. Cellular properties to be used as the indicator include anchor dependency, cytokine-dependent response, hormone dependency, drug resistance, cell motility, cell migration activity, pulsatory activity, and alteration in intracellular substances. Cell motility includes cell infiltration activity and cell migration activity. The alterations in intracellular substances include, for example, alterations in enzyme activity, mRNA levels, levels of intracellular signaling molecules such as Ca^{2+} and cAMP, and intracellular protein levels. When a cell membrane receptor is used, alterations in the cell

proliferating activity induced by receptor stimulation can be used as the indicator. The indicators to be used in tissue-based assays include functional alterations adequate for the subject tissue. In *in vivo* assays, alterations in tissue weight, alterations in the blood system (for example, alterations in blood cell counts, protein contents, or enzyme activities), alterations in electrolyte levels, and alterations in the circulating system (for example, alterations in blood pressure or heart rate).

[0082]

The methods for measuring such detection indices are not particularly limited. For example, absorbance, luminescence, color development, fluorescence, radioactivity, fluorescence polarization, surface plasmon resonance signal, time-resolved fluorescence, mass, absorption spectrum, light scattering, and fluorescence resonance energy transfer may be used. These measurement methods are known to those skilled in the art and may be selected appropriately depending on the purpose. For example, absorption spectra can be obtained by using a conventional photometer, plate reader, or such; luminescence can be measured with a luminometer or such; and fluorescence can be measured with a fluorometer or such. Mass can be determined with a mass spectrometer. Radioactivity can be determined with a device such as a gamma counter depending on the type of radiation. Fluorescence polarization can be measured with BEACON (TaKaRa). Surface plasmon resonance signals can be obtained with BIACORE. Time-resolved fluorescence, fluorescence resonance energy transfer, or such can be measured with ARVO or such. Furthermore, a flow cytometer can also be used for measuring. It is possible to use one of the above methods to measure two or more different types of detection indices. A greater number of detection indices may also be examined by using two or more measurement methods simultaneously and/or consecutively. For example, fluorescence and fluorescence resonance energy transfer can be measured at the same time with a fluorometer.

[0083]

The present invention provides pharmaceutical compositions comprising antibodies of this invention. The pharmaceutical compositions comprising antibodies of the present invention are useful for treating and/or preventing thrombocytopenia and such. Time required for the platelet count to recover to the normal level can be shortened by administering an antibody of the present invention after donation of platelet components. The amount of platelet components at the time of blood collection can be increased by pre-administering an antibody of the present invention.

[0084]

When used as pharmaceutical compositions, the antibodies of the present invention can be formulated by methods known to those skilled in the art. For example, the antibodies can be

administered parenterally by injection of a sterile solution or suspension in water or other pharmaceutically acceptable solvents. For example, the antibodies can be formulated by appropriately combining with pharmaceutically-acceptable carriers or solvents, specifically, sterile water or physiological saline, vegetable oils, emulsifiers, suspending agents, surfactants, stabilizers, flavoring agents, excipients, vehicles, preservatives, binding agents, and such, and mixing at a unit dosage and form required by accepted pharmaceutical implementations. In such formulations, the amount of the thus obtained active ingredient should be within the required range.

[0085]

A sterile composition to be injected can be formulated using a vehicle such as distilled water used for injection, according to standard protocols.

[0086]

Aqueous solutions used for injections include, for example, physiological saline and isotonic solutions comprising glucose or other adjunctive agents such as D-sorbitol, D-mannose, D-mannitol, and sodium chloride. They may also be combined with an appropriate solubilizing agent such as alcohol, specifically, ethanol, polyalcohol such as propylene glycol or polyethylene glycol, or non-ionic detergent such as polysorbate 80TM or HCO-50, as necessary.

[0087]

Oil solutions include sesame oils and soybean oils, and can be combined with solubilizing agents such as benzyl benzoate or benzyl alcohol. Injection solutions may also be formulated with buffers, for example, phosphate buffers or sodium acetate buffers; analgesics, for example, procaine hydrochloride; stabilizers, for example, benzyl alcohol or phenol; or anti-oxidants. The prepared injections are typically aliquoted into appropriate ampules.

[0088]

The administration is preferably carried out parenterally, specifically, by injection, intranasal administration, intrapulmonary administration, percutaneous administration, or such. Injections include, for example, intravenous injections, intramuscular injections, intraperitoneal injections, and subcutaneous injections. The injection solutions can be also administered systemically or locally.

[0089]

The administration methods can be selected properly according to the patient's age, condition, and such. The applied dose of a pharmaceutical composition comprising an antibody or polynucleotide encoding the antibody may be, for example, in the range of 0.0001 to 1,000 mg/kg body weight. Alternatively, the dosage may be, for example, in the range of 0.001 to 100,000 mg/kg body weight. However, the dosage is not restricted to the values described above. The dosage and administration methods depend on the patient's weight, age, and

condition, and are appropriately selected by those skilled in the art.

[Examples]

[0090]

The present invention is specifically illustrated below with reference to Examples, but it is not to be construed as being limited thereto.

[Example 1] Preparation of anti-human Mpl antibodies

1.1 Establishment of Mpl-expressing BaF3 cell lines

BaF3 cell lines expressing the full-length Mpl gene were established to obtain cell lines that proliferate in a TPO-dependent manner.

A full-length human Mpl cDNA (Palacios, R. *et al.*, Cell (1985) 41: 727-734) (GenBank accession NO. NM_005373) was amplified by PCR. The cDNA was cloned into a pCOS2 expression vector to construct pCOS2-hMplfull. The expression vector pCOS2 was constructed by removing the DHFR gene expression region from pCHOI (Hirata, Y. *et al.*, FEBS Letter (1994) 356: 244-248), where the expression region of the neomycin resistance gene HEF-VH-gyl (Sato, K. *et al.*, Mol Immunol. (1994) 31: 371-381) is inserted.

The cynomolgus monkey Mpl cDNA (SEQ ID NO: 164) was cloned from total RNA extracted from the bone marrow cells of cynomolgus monkey, using a SMART RACE cDNA Amplification Kit (Clontech). The resulting cynomolgus monkey cDNA was inserted into pCOS2 to construct pCOS2-monkeyMplfull.

Then, the full-length mouse Mpl cDNA (GenBank accession NO. NM_010823) was amplified by PCR, and inserted into pCOS2 to construct pCOS2-mouseMplfull.

Each vector (20 µg) prepared as described above was mixed with BaF3 cells (1×10^7 cells/mL) suspended in PBS in Gene Pulser cuvettes. This mixture was then pulsed at 0.33 kV and 950 µFD using a Gene Pulser II (Bio-Rad). The BaF3 cells introduced with the above DNAs by electroporation were added to RPMI 1640 medium (Invitrogen) containing 1 ng/mL mouse interleukin 3 (hereinafter abbreviated as mIL-3; Peprotech), 500 µg/mL Geneticin (Invitrogen), and 10% FBS (Invitrogen), and selected to establish a human Mpl-expressing BaF3 cell line (hereinafter abbreviated as "BaF3-human Mpl"), monkey Mpl-expressing BaF3 cell line (hereinafter abbreviated as BaF3-monkey Mpl), and mouse Mpl-expressing BaF3 cell line (hereinafter abbreviated as "BaF3-mouse Mpl"). Following selection, these cells were cultured and maintained in RPMI 1640 containing 1 ng/mL rhTPO (R&D) and 10% FBS.

[0091]

1.2 Establishment of Mpl-expressing CHO cell lines

CHO cell lines expressing the full-length Mpl gene were established to obtain cell lines to be used for assessing binding activity by flow cytometry.

First, the DHFR gene expression site from pCHOI was inserted into pCXN2 (Niwa, H.

et al., Gene (1991) 108: 193-199) at the HindIII site to prepare a pCXND3 expression vector. The respective Mpl genes were amplified by PCR using pCOS2-hMplfull, pCOS2-monkeyMplfull, and pCOS2-mouseMplfull as templates, and primers with a His-tag sequence. The PCR products were cloned into pCXND3 to construct pCXND3-hMpl-His, pCXND3-monkey Mpl-His, and pCXND3-mouse Mpl-His, respectively.

Vectors thus prepared (25 µg each) were mixed with a PBS suspension of CHO-DG44 cells (1×10^7 cells/mL) in Gene Pulser cuvettes. The mixture was then pulsed at 1.5 kV and 25 µFD using Gene Pulser II (Bio-Rad). The CHO cells introduced with these DNAs by electroporation were added to CHO-S-SFMII medium (Invitrogen) containing 500 µg/mL Geneticin and 1x HT (Invitrogen). A human Mpl-expressing CHO cell line (hereinafter abbreviated as "CHO-human Mpl"), monkey Mpl-expressing CHO cell line (hereinafter abbreviated as "CHO-monkey Mpl"), and mouse Mpl-expressing CHO cell line (hereinafter abbreviated as "CHO-mouse Mpl") were established through selection.

[0092]

1.3 Preparation of soluble human Mpl protein

To prepare soluble human Mpl protein, an expression system using insect Sf9 cells for production and secretion of the protein was constructed as described below.

A DNA construct encoding the extracellular region of human Mpl (Gln 26 to Trp 491) with a downstream FLAG tag was prepared. The construct was inserted into a pBACSurf-1 Transfer Plasmid (Novagen) between the *Pst*I and *Sma*I sites to prepare pBACSurf1-hMpl-FLAG. Then, Sf9 cells were transformed with 4 µg of pBACSurf1-hMpl-FLAG using the Bac-N-Blue Transfection Kit (Invitrogen). The culture supernatant was collected after three-day incubation. Recombinant virus was isolated by plaque assays. The prepared virus stock was used to infect Sf9 cells, and the culture supernatant was collected.

Soluble human Mpl protein was purified from the obtained culture supernatant as described below. The culture supernatant was loaded onto a Q Sepharose Fast Flow (Amersham Biosciences) for adsorption, and the adsorbed protein was then eluted with 50 mM Na-phosphate buffer (pH7.2) containing 0.01% (v/v) Tween20 and 500 mM NaCl. After the eluates were loaded onto a FLAG M2-Agarose (Sigma-Aldrich) for adsorption, the protein adsorbed was eluted with 100 mM glycine-HCl buffer (pH3.5) containing 0.01% (v/v) Tween20. Immediately after elution, the fraction obtained was neutralized with 1 M Tris-Cl (pH8.0) and the buffer was exchanged with PBS (-) and 0.01% (v/v) Tween20 using PD-10 columns (Amersham Biosciences). The purified soluble Mpl protein was referred to as "shMpl-FLAG".

[0093]

1.4 Preparation of human Mpl-IgG Fc fusion protein

Human fusion protein Mpl-IgG Fc gene was prepared according to the method by

Bennett *et al.* (Bennett, B. D. *et al.*, J. Biol. Chem. (1991) 266: 23060-23067). A nucleotide sequence encoding the extracellular region of human Mpl (Gln 26 to Trp 491) was linked to a nucleotide sequence encoding the Fc region of human IgG- γ 1 (a region downstream of Asp 216). A *Bst*EII sequence (amino acids: Val-Thr) was attached to the junction as a fusion linker between these two regions. A 19-amino acid signal peptide derived from human IgG H chain variable region was used as the signal sequence. The resulting human fusion protein Mpl-IgG Fc gene was cloned into pCXND3 to construct pCXND3-hMpl-Fc.

The vector thus prepared (25 μ g) was mixed with a PBS suspension of CHO-DG44 cells (1×10^7 cells/mL) in Gene Pulser cuvettes. The mixture was then pulsed at 1.5 kV and 25 μ FD using Gene Pulser II (Bio-Rad). The CHO cells introduced with the DNA by electroporation were added to CHO-S-SFMII medium containing 500 μ g/mL Geneticin and 1x HT (Invitrogen). shMPL-Fc-expressing CHO cell line (CHO-hMpl-Fc) was then established through selection.

Human Mpl-IgG Fc fusion protein was purified from the culture supernatant as described below.

The culture supernatant was loaded onto a Q Sepharose Fast Flow (Amersham Biosciences) for adsorption, and then the adsorbed protein were eluted with 50 mM Na-phosphate buffer (pH7.6) containing 0.01% (v/v) Tween20 and 1 M NaCl. After the eluates were loaded onto a HiTrap protein G HP column (Amersham Biosciences) for adsorption, the adsorbed protein was eluted with 0.1 M glycine-HCl buffer (pH2.7) containing 150 mM NaCl and 0.01% (v/v) Tween20. Immediately after elution, the obtained fraction was neutralized with 1 M Tris-Cl (pH8.0) and the buffer was exchanged with PBS (-) and 0.01% (v/v) Tween20 using PD-10 columns (Amersham Biosciences). The purified soluble Mpl protein was referred to as "hMpl-Fc".

[0094]

1.5 Immunization with shMpl-FLAG and hybridoma selection

MRL/MpJUmmCrj-lpr/lpr mice (hereinafter abbreviated as "MRL/lpr mice"; purchased from Charles River, Japan) were immunized; the primary immunization was carried out at eight weeks of age. For every single mouse, an emulsion containing 100 μ g of shMPL-FLAG combined with Freund's complete adjuvant (H37 Ra; Beckton Dickinson), was administered subcutaneously as the primary injection. As a booster injection, an emulsion containing shMPL-FLAG (50 μ g per mouse) combined with Freund's incomplete adjuvant (Beckton Dickinson) was administered subcutaneously. Three mice which have been immunized six times in total were subjected to a final injection of shMPL-FLAG (50 μ g per mouse) through the caudal vein. Cell fusion was achieved by mixing the mouse myeloma P3-X63Ag8U1 cells (P3U1; purchased from ATCC) and mouse splenocytes using polyethylene glycol 1500 (Roche Diagnostics). Hybridoma selection in HAT medium began the following day and culture

supernatants were obtained. Screening was carried out by ELISA, using immunoplates immobilized with shMpl-FLAG or hMpl-Fc and the assayed cell growth activity of BaF3-hMpl as an index. Positive clones were isolated as single clones by limiting dilution and then cultured in a large scale. The culture supernatants were collected.

5 [0095]

1.6 Analyses of anti-human Mpl antibodies

Antibody concentrations were determined by carrying out a mouse IgG sandwich ELISA using goat anti-mouse IgG (gamma) (ZYMED) and alkaline phosphatase-goat anti-mouse IgG (gamma) (ZYMED), generating a calibration curve by GraphPad Prism (GraphPad Software; USA), and calculating the antibody concentrations from the calibration curve. Commercially available antibodies of the same isotype were used as standards.

[0096]

Antibody isotypes were determined by antigen-dependent ELISA using isotype-specific secondary antibodies. hMpl-Fc was diluted to 1 $\mu\text{g/mL}$ with a coating buffer (0.1 mM NaHCO_3 , pH9.6) containing 0.02% (w/v) NaN_3 , and then added to ELISA plates. The plates were incubated overnight at 4°C for coating. The plates were blocked with a diluent buffer (50 mM Tris-HCl (pH8.1) containing 1 mM MgCl_2 , 150 mM NaCl, 0.05% (v/v) Tween20, 0.02% (w/v) NaN_3 , 1% (w/v) BSA). After the addition of hybridoma culture supernatants, the plates were allowed to stand at room temperature for 1 hr. After washing with a rinse buffer (0.05% (v/v) Tween20 in PBS), alkaline phosphatase-labeled isotype-specific secondary antibodies were added to the plates. Then, the plates were allowed to stand at room temperature for 1 hr. Color development was carried out using SIGMA104 (Sigma-Aldrich) diluted to 1 mg/mL with a substrate buffer (50 mM NaHCO_3 , pH9.8) containing 10 mM MgCl_2 , and absorbance was measured at 405 nm using Benchmark Plus (Bio-Rad).

25 [0097]

The binding activities of an antibody to shMpl-FLAG and hMPL-Fc were determined by ELISA. ELISA plates were coated with 1 $\mu\text{g/mL}$ of purified shMpl-FLAG or hMPL-Fc, and blocked with a diluent buffer. Hybridoma culture supernatants were added to the plates, and the plates were allowed to stand at room temperature for 1 hr. Then, alkaline phosphatase-labeled anti-mouse IgG antibodies (Zymed) were added to the plates. Color development was similarly carried out using the above method. Following a one-hour coloring reaction at room temperature, absorbance was measured at 405 nm and EC_{50} values were computed using GraphPad Prism.

[0098]

35 CHO-human Mpl cells and CHO-monkey Mpl cells were harvested, and suspended in FACS Buffer (1% FBS/ PBS) to a final concentration of 1×10^6 cells/mL. The suspensions

were aliquoted into Multiscreen (Millipore) at 100 μ L/well, and the culture supernatants were removed by centrifugation. Culture supernatants diluted to 5 μ g/mL were added to the plates and incubated on ice for 30 min. The cells were washed once with FACS buffer, and incubated on ice for 30 min following the addition of an FITC-labeled anti-mouse IgG antibody (Beckman Coulter). After incubation, the mixture was centrifuged at 500 rpm for 1 min. The supernatants were removed, and then the cells were suspended in 400 μ L of FACS buffer. The samples were analyzed by flow cytometry using EPICS ELITE ESP (Beckman Coulter). An analysis gate was set on the forward and side scatters of a histogram to include viable cell populations.

[0099]

Agonistic activities of an antibody were evaluated using BaF3-human Mpl and BaF3-monkey Mpl which proliferate in a TPO-dependent manner. Cells of each cell line were suspended at 4×10^5 cells/ml in RPMI 1640/10% FBS (Invitrogen), and each suspension was aliquoted into a 96-well plate at 60 μ L/well. A 40- μ L aliquot of rhTPO (R&D) and hybridoma culture supernatants prepared at various concentrations was added into each well. The plates were then incubated at 37°C under 5% CO₂ for 24 hr. A 10- μ L aliquot of the Cell Count Reagent SF (Nacalai Tesque) was added into each well. After incubation for 2 hr, absorbance was measured at 450 nm (and at 655 nm as a control) using a Benchmark Plus. EC₅₀ values were calculated using GraphPad Prism.

The above analysis yielded a total of 163 clones of mouse monoclonal antibodies that bind to human Mpl.

[0100]

1.7 Purification of anti-human Mpl antibodies

Anti-human Mpl antibodies were purified from hybridoma culture supernatants as described below.

After the culture supernatants were loaded onto HiTrap protein G HP columns (Amersham Biosciences) for adsorption, the antibodies were eluted with 0.1 M glycine-HCl (pH2.7). Immediately after elution, the fractions were neutralized with 1 M Tris-Cl (pH9.0), dialyzed against PBS for one day, and the buffer was replaced.

[0101]

1.8 Determination of epitopes for the anti-human Mpl antibody VB22B

Since the anti-human Mpl antibody VB22B can be used for Western blotting, a GST-fusion protein containing a partial sequence of human Mpl was constructed for VB22B epitope analysis. MG1 (Gln26 to Trp491) and MG2 (Gln26 to Leu274) regions were each amplified by PCR, and cloned into pGEX-4T-3 (Amersham Biosciences) to be expressed as GST fusion proteins. The resulting plasmid DNAs were transformed into DH5 α to give

transformants. A final concentration of 1 mM IPTG was added to the transformants in their logarithmic growth phase to induce the expression of GST fusion proteins. The bacterial cells were harvested after two hours of incubation. The cells were lysed by sonication. The lysates were centrifuged in XL-80 Ultracentrifuge (Beckman, Rotor 70.1Ti) at 35,000 rpm for 30 min. The culture supernatants were removed, and then the fusion proteins were purified using GST Purification Modules (Amersham Biosciences). The samples were separated by 10%-SDS-PAGE, and then transferred onto a PVDF membrane. The membrane was Western blotted with the murine antibody VB22B. VB22B was found to recognize both MG-1 and MG-2, indicating that the VB22B epitope is located in the (Gln26 to Leu274) region.

[0102]

Then, GST fusion proteins containing the respective regions of human Mpl: MG3 (Gln26 to Ala189), MG4 (Gln26 to Pro106), MG5 (Gln26 to Glu259), and MG6 (Gln26 to Gly245) were prepared and analyzed by Western blotting using the same procedure described above. VB22B was found to recognize MG5 and MG6, but not MG3 and MG4. This suggests that the VB22B epitope is located within the (Ala189 to Gly245) region. In addition, GST was fused with MG7 (Gln26 to Ala231) and MG8 (Gln26 to Pro217) to prepare GST fusion proteins. VB22B recognized MG7 but not MG8, suggesting that the VB22B epitope is located in the (Gln217 to Ala231) region. Furthermore, GST fusion protein containing MG10 (Gln213 to Ala231) was recognized by VB22B, suggesting that the VB22B epitope is located within the limited region of 19 amino acids between Gln213 and Ala231.

[0103]

1.9 Kinetic analyses of the antigen-antibody reaction for anti-human Mpl antibody VB22B

Since the anti-human Mpl antibody VB22B binds to soluble recombinant Mpl, kinetic analyses of the antigen-antibody reaction between VB22B IgG and human Mpl-IgG Fc fusion protein were carried out as described in Example 1.4. The Sensor Chip CM5 (Biacore) was placed in Biacore 2000 (Biacore), and human Mpl-IgG Fc fusion protein was immobilized onto the chip by amine-coupling methods. Then, 1.25 to 20 $\mu\text{g/mL}$ of VB22B IgG solution was prepared using HBS-EP Buffer (Biacore), and injected over the chip surface for 2 min to reveal the binding region. Then, HBS-EP Buffer was injected over the chip surface for 2 min to reveal the dissociation region. VB22B IgG bound to the human Mpl-IgG Fc fusion protein on the sensor chip was removed by injecting 10 mM NaOH over the sensor chip for 15 sec, and the chip was recovered. HBS-EP Buffer was used as the running buffer, and the flow rate was 20 $\mu\text{L/min}$. Using the BIAevaluation Version 3.1 (Biacore) software, the reaction rate constant at each concentration was calculated from the sensorgrams. The dissociation constant (K_D) for VB22B IgG was determined to be $1.67 \pm 0.713 \times 10^{-9}$ M.

[0104]

[Example 2] Preparation of single-chain anti-human Mpl antibodies

Among the prepared anti-human Mpl antibodies, 23 types of antibodies, which exhibit higher binding activities and agonistic activities, were selected to construct expression systems for single-chain antibodies using genetic engineering techniques. An exemplary method for constructing a single-chain antibody derived from the anti-human Mpl antibody VB22B is described below.

[0105]

2.1 Cloning of the anti-human Mpl antibody variable region

The variable region was amplified by RT-PCR using total RNA extracted from hybridomas producing anti-human Mpl antibodies. Total RNA was extracted from 1×10^7 hybridoma cells using the RNeasy Plant Mini Kit (QIAGEN).

[0106]

A 5'-terminal fragment of the gene was amplified from 1 μ g of total RNA by the SMART RACE cDNA Amplification Kit (Clontech), using a synthetic oligonucleotide MHC-IgG2b (SEQ ID NO: 166) complementary to mouse IgG2b constant region or a synthetic oligonucleotide kappa (SEQ ID NO: 167) complementary to mouse κ chain constant region. Reverse transcription was carried out at 42°C for 1.5 hr.

[0107]

The composition of the PCR reaction solution (50 μ L in total) is shown below.

10x Advantage 2 PCR Buffer (Clontech)	5 μ L
10x Universal Primer A Mix (Clontech)	5 μ L
dNTPs (dATP, dGTP, dCTP, and dTTP) (Clontech)	0.2 mM
Advantage 2 Polymerase Mix (Clontech)	1 μ L
Reverse transcription product	2.5 μ L
Synthetic oligonucleotide, MHC-IgG2b or kappa	10 pmol

[0108]

The PCR reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 5 sec and 72°C for 3 min;

five cycles of 94°C for 5 sec, 70°C for 10 sec, and 72°C for 3 min;

25 cycles of 94°C for 5 sec, 68°C for 10 sec, and 72°C for 3 min;

and final extension was at 72°C for 7 min.

[0109]

The PCR products were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and cloned into a pGEM-T Easy Vector (Promega). The nucleotide sequence was then determined using the ABI 3700 DNA Analyzer (Perkin Elmer).

[0110]

The nucleotide sequence of cloned VB22B H chain variable region (hereinafter abbreviated as "VB22B-VH") is shown in SEQ ID NO: 117, and its amino acid sequence is shown in SEQ ID NO: 118. The nucleotide sequence of the L chain variable region (hereinafter abbreviated as "VB22B-VL") is shown in SEQ ID NO: 119, and its amino acid sequence is shown in SEQ ID NO: 120.

[0111]

2.2 Preparation of expression vectors for anti-human Mpl diabodies

The gene encoding VB22B single-chain Fv (hereinafter abbreviated as "VB22B diabody") containing a five-amino acid linker sequence was constructed, by linking a nucleotide sequence encoding a (Gly4Ser)₁ linker to the VB22B-VH-encoding gene at its 3' end and to the VB22B-VL-encoding gene at its 5' end; both of which have been amplified by PCR.

[0112]

The VB22B-VH forward primer, 70·115HF, (SEQ ID NO: 168) was designed to contain an *Eco*RI site. The VB22B-VH reverse primer, 33·115HR, (SEQ ID NO: 169) was designed to hybridize to a DNA encoding the C terminus of VB22B-VH, and to have a nucleotide sequence encoding the (Gly4Ser)₁ linker and a nucleotide sequence hybridizing to the DNA encoding the N terminus of VB22B-VL. The VB22B-VL forward primer, 33·115LF, (SEQ ID NO: 170) was designed to have a nucleotide sequence encoding the N terminus of VB22B-VL, a nucleotide sequence encoding the (Gly4Ser)₁ linker, and a nucleotide sequence encoding the C terminus of VB22B-VH. The VB22B-VL reverse primer, 33·115LR, (SEQ ID NO: 171) was designed to hybridize to a DNA encoding the C terminus of VB22B-VL and to have a nucleotide sequence encoding a FLAG tag (Asp Tyr Lys Asp Asp Asp Asp Lys/SEQ ID NO: 172) and a *Not*I site.

[0113]

In the first round of PCR, two PCR products: one containing VB22B-VH and a linker sequence, and the other containing VB22B-VL and the identical linker sequence, were synthesized by the procedure described below.

The composition of the PCR reaction solution (50 µL in total) is shown below.

10x PCR Buffer (TaKaRa)	5 µL
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
pGEM-T Easy vector comprising VB22B-VH or VB22B-VL gene	10 ng
Synthetic oligonucleotides, 70·115HF and 33·115HR, or 33·115LF and 33·115LR	10 pmol

[0114]

The PCR reaction conditions were:

94°C (initial temperature) for 30 sec;
 five cycles of: 94°C for 15 sec and 72°C for 2 min;
 five cycles of 94°C for 15 sec and 70°C for 2 min;
 28 cycles of 94°C for 15 sec and 68°C for 2 min;
 and final extension was at 72°C for 5 min.

[0115]

After the PCR products of about 400 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), the second-round PCR was carried out using aliquots of the respective PCR products according to the protocol described below.

The composition of the PCR reaction solution (50 µL in total) is shown below.

10x PCR Buffer (TaKaRa)	5 µL
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 unit
First-round PCR products (two types)	1 µL
Synthetic oligonucleotides, 70-115HF and 33-115LR	10 pmol

[0116]

The reaction conditions were:

94°C (initial temperature) for 30 sec;
 five cycles of 94°C for 15 sec and 72°C for 2 min;
 five cycles of 94°C for 15 sec and 70°C for 2 min;
 28 cycles of 94°C for 15 sec and 68°C for 2 min;
 and final extension was at 72°C for 5 min.

[0117]

The PCR products of about 800 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and then digested with *Eco*RI and *Not*I (both from TaKaRa). The resulting DNA fragments were purified using the QIAquick PCR Purification Kit (QIAGEN), and then cloned into pCXND3 to prepare pCXND3-VB22B db.

[0118]

2.3 Preparation of expression vectors for anti-human Mpl antibody sc(Fv)₂

To prepare expression plasmids for the modified antibody [sc(Fv)₂] comprising two units of H chain variable region and two units of L chain variable region derived from VB22B, the above-described pCXND3-VB22B db was modified by PCR using the procedure shown below. The process for constructing the sc(Fv)₂ gene is illustrated in Fig. 1.

[0119]

First, PCR method was carried out to amplify (a) the VB22B-VH-encoding gene in which a nucleotide sequence encoding a 15-amino acid linker (Gly₄Ser)₃ was added to its 3' end;

and (b) the VB22B-VL-encoding gene containing the identical linker nucleotide sequence added to its 5' end. The desired construct was prepared by linking these amplified genes. Three new primers were designed in this construction process. The VB22B-VH forward primer, VB22B-fpvu, (primer A; SEQ ID NO: 173) was designed to have an *EcoRI* site at its 5' end and to convert Gln22 and Leu23 of VB22B db into a *PvuII* site. The VB22B-VH reverse primer, sc-rL15, (primer B; SEQ ID NO: 174) was designed to hybridize to a DNA encoding the C terminus of VB22B-VH, and to have a nucleotide sequence encoding the (Gly₄Ser)₃ linker, as well as a nucleotide sequence hybridizing to a DNA encoding the N terminus of VB22B-VL. The VB22B-VL forward primer, sc-fL15, (primer C; SEQ ID NO: 175) was designed to have a nucleotide sequence encoding the N terminus of VB22B-VL, a nucleotide sequence encoding the (Gly₄Ser)₃ linker, and a nucleotide sequence encoding the C terminus of VB22B-VH.

[0120]

In the first-round PCR, two PCR products: one comprising VB22B-VH and a linker sequence, and the other comprising VB22B-VL and the identical linker sequence, were synthesized by the procedure described below.

The composition of the PCR reaction solution (50 μ L in total) is shown below.

10x PCR Buffer (TaKaRa)	5 μ L
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
pCXND3-VB22B db	10 ng
Synthetic oligonucleotides, VB22B-fpvu, sc-rL15 or sc-fL15, and 33-115LR (primer D)	10 pmol

[0121]

The reaction conditions were:

94°C (initial temperature) for 30 sec;
 five cycles of 94°C for 15 sec and 72°C for 2 min;
 five cycles of 94°C for 15 sec and 70°C for 2 min;
 28 cycles of 94°C for 15 sec and 68°C for 2 min;
 and final extension was at 72°C for 5 min.

[0122]

After the PCR products of about 400 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), the second-round PCR was carried out using aliquots of the respective PCR products according to the protocol described below.

The composition of the PCR reaction solution (50 μ L in total) is shown below.

10x PCR Buffer (TaKaRa)	5 μ L
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM

DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
First-round PCR product (two types)	1 μ L
Synthetic oligonucleotide, 70·115HF and 33·115LR	10 pmol

[0123]

The reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 15 sec and 72°C for 2 min;

5 five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

[0124]

10 The PCR products of about 800 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and then digested with *Eco*RI and *Not*I (both from TaKaRa). The resulting DNA fragments were purified using the QIAquick PCR Purification Kit (QIAGEN), and then cloned into pBacPAK9 (Clontech) to construct pBacPAK9-scVB22B.

[0125]

A fragment to be inserted into the *Pvu*II site of pBacPAK9-scVB22B was prepared.

15 Specifically, the fragment has a *Pvu*II recognition site at both ends and a nucleotide sequence, in which a gene encoding the VB22B-VH N-terminus is linked, via a (Gly₄Ser)₃ linker-encoding nucleotide sequence, to a gene encoding the amino acid sequence of an N terminus-deleted VB22B-VH linked to VB22B-VL via the (Gly₄Ser)₃ linker. Two primers were newly designed to prepare the fragment by PCR. The forward primer for the fragment of interest, Fv2-f (primer
20 E; SEQ ID NO: 176), was designed to have a *Pvu*II site at its 5' end and a VB22B-VH 5'-end sequence. The reverse primer for the fragment of interest, Fv2-r (primer F; SEQ ID NO: 177), was designed to hybridize to a DNA encoding the C terminus of VB22B-VL, and to have a *Pvu*II site, a nucleotide sequence encoding the (Gly₄Ser)₃ linker, and a nucleotide sequence hybridizing to a DNA encoding the N terminus of VB22B-VH. PCR was carried out using
25 pBacPAK9-scVB22B as a template as described below.

[0126]

The composition of the PCR reaction solution (50 μ L in total) is shown below.

10x PCR Buffer (TaKaRa)	5 μ L
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
pBacPAK9-scVB22B	10 μ g
Synthetic oligonucleotide, Fv2-f and Fv2-r	10 pmol

[0127]

The reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 15 sec and 72°C for 2 min;

five cycles of 94°C for 15 sec and 70°C for 2 min;

5 28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

[0128]

The PCR products of about 800 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and then cloned into the pGEM-T Easy Vector (Promega).

10 After sequencing, the plasmid was digested with *PvuII* (TaKaRa), and the fragment of interest was recovered. The recovered fragment was ligated to pBacPAK9-scVB22B pre-digested with *PvuII* (TaKaRa) to construct pBacPAK9-VB22B sc(Fv)₂. After the resulting vector was digested with *EcoRI* and *NotI* (both from TaKaRa), the fragment of about 1,800 bp was purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN). The fragment was then
15 cloned into a pCXND3 expression vector to construct pCXND3-VB22B sc(Fv)₂.

[0129]

2.4 Expression of single-chain anti-human Mpl antibody in animal cells

A cell line stably expressing the single-chain antibody was prepared from CHO-DG44 cells as described below. Gene transfer was achieved by electroporation using a Gene Pulser II
20 (Bio-Rad). An expression vector (25 µg) and 0.75 mL of CHO-DG44 cells suspended in PBS (1 x 10⁷ cells/mL) were mixed. The resulting mixture was cooled on ice for 10 min, transferred into a cuvette, and pulsed at 1.5-kV and 25 µFD. After a ten-minute restoration period at room temperature, the electroporated cells were plated in CHO-S-SFMII medium (Invitrogen) containing 500 µg/mL Geneticin (Invitrogen). CHO cell lines expressing the single-chain
25 antibody were established through selection. A cell line stably expressing VB22B sc(Fv)₂ and its culture supernatants were obtained by this method.

[0130]

The transient expression of the single-chain antibody was achieved using COS7 cells as described below. An expression vector (10 µg) and 0.75 mL of CHO-DG44 cells suspended in
30 PBS (1 x 10⁷ cells/mL) were mixed. The resulting mixture was cooled on ice for 10 min, transferred into a cuvette, and then pulsed at 1.5-kV and 25 µFD. After a ten-minute restoration period at room temperature, the electroporated cells were plated in DMEM/10% FBS medium (Invitrogen). The cells were incubated overnight and then washed with PBS. CHO-S-SFMII medium was added and the cells were cultured for about three days. The culture supernatants
35 for preparing the VB22B diabody were thus prepared.

[0131]

2.5 Quantitation of single-chain anti-human Mpl antibodies in culture supernatants

The culture supernatant concentration of the single-chain anti-human Mpl antibody transiently expressed in COS cells was determined using surface plasmon resonance. A sensor chip CM5 (Biacore) was placed in Biacore 2000 (Biacore). ANTI-FLAG® M2 Monoclonal Antibody (Sigma-Aldrich) was immobilized onto the chip. An appropriate concentration of sample was injected over the chip surface at a flow rate of 5 mL/sec, and 50 mM diethylamine was used to dissociate the bound antibody. Changes in the mass during sample injection were recorded, and the sample concentration was calculated from the calibration curve prepared using the mass changes of a standard sample. db12E10 (see WO 02/33073 and WO 02/33072) was used as the diabody standard, and 12E10 sc(Fv)₂ which has the same gene structure as that of sc(Fv)₂ was used as the sc(Fv)₂ standard.

[0132]

2.6 Purification of anti-human Mpl single-chain antibodies

The culture supernatants of VB22B diabody-expressing COS7 cells or CHO cells was loaded onto an Anti-Flag M2 Affinity Gel (Sigma-Aldrich) column equilibrated with a 50 mM Tris-HCl buffer (pH7.4) containing 150 mM NaCl and 0.05% Tween20. The absorbed antibodies were eluted with 100 mM glycine-HCl (pH3.5). The fractions eluted were immediately neutralized with 1 M Tris-HCl (pH8.0), and loaded onto a HiLoad 26/60 Superdex 200 pg (Amersham Biosciences) column for gel filtration chromatography. PBS/0.01% Tween20 was used in the gel filtration chromatography.

[0133]

VB22B sc(Fv)₂ was purified from the culture supernatants of VB22B sc(Fv)₂-expressing COS7 cells or CHO cells under the same conditions used for purifying the diabodies. A large-scale preparation of VB22B sc(Fv)₂ was prepared by loading the CHO cell culture supernatants onto a Macro-Prep Ceramic Hydroxyapatite Type I (Bio-Rad) column equilibrated with a 20 mM phosphate buffer (pH6.8), and eluting the VB22B sc(Fv)₂ in a stepwise manner with 250 mM phosphate buffer (pH6.8). The eluted fraction was concentrated on an ultrafilter, and then fractionated by gel filtration chromatography using a HiLoad 26/60 Superdex 200 pg (Amersham Biosciences) column, and a fraction corresponding to the molecular weight range of about 40 kD to 70 kD was obtained. The fraction was loaded onto an Anti-Flag M2 Affinity Gel column equilibrated with a 50 mM Tris-HCl buffer (pH7.4) containing 150 mM NaCl and 0.05% Tween20. The absorbed antibody was eluted with 100 mM glycine-HCl (pH3.5). The eluted fraction was immediately neutralized with 1 M Tris-HCl (pH8.0), and loaded onto a HiLoad 26/60 Superdex 200 pg (Amersham Biosciences) column for gel filtration chromatography. 20 mM acetate (pH6.0) containing 150 mM NaCl and 0.01% Tween80 was used in the gel filtration chromatography.

[0134]

2.7 Binding activity analyses of single-chain anti-human Mpl antibodies by flow cytometry

CHO-human Mpl, CHO-monkey Mpl, and CHO-mouse Mpl cells were recovered and suspended in FACS buffer (1% FBS/PBS) to a final concentration of 1×10^6 cells/mL. Cell suspensions were aliquoted at 100- μ L/well into the Multiscreen-HV Filter Plates (Millipore). After centrifugation, the supernatant was removed. An appropriate concentration of diabody or sc(Fv)₂ was added into each well and incubated on ice for 30 min. The cells were washed once with 200 μ L of FACS buffer, and incubated on ice for 30 min following the addition of 10 μ g/mL ANTI-FLAG® M2 Monoclonal Antibody (Sigma-Aldrich). The cells were then washed once with 200 μ L of FACS buffer, and a 100x-diluted FITC-labeled anti-mouse IgG antibody (Beckman Coulter) was added to the plate. The plate was incubated on ice for 30 min. After centrifugation, the supernatant was removed. The cells were suspended in 400 μ L of FACS Buffer, and then analyzed by flow cytometry using EPICS ELITE ESP (Beckman Coulter). An analysis gate was set on the forward and side scatters of a histogram to include viable cell populations.

[0135]

The binding activity of the purified VB22B sc(Fv)₂ to various Mpl molecules expressed in CHO cells was determined (Fig. 2). VB22B sc(Fv)₂ was found to specifically bind to CHO-human Mpl and CHO-monkey Mpl but not to the host cell CHO or CHO-mouse Mpl. This binding characteristic of VB22B sc(Fv)₂ is comparable to those of VB22B IgG, indicating that the antibody binding site remains unaltered by reduction of molecular weight.

[0136]

2.8 Analyses of TPO-like agonistic activity for single-chain anti-human Mpl antibodies

TPO-like agonistic activity was assessed using BaF3-human Mpls or BaF3-monkey Mpls that proliferate in a TPO-dependent manner.

[0137]

Cells from each cell line were washed twice with RPMI 1640/1% FBS (fetal bovine serum) (Invitrogen), and then suspended in RPMI 1640/10% FBS to a concentration of 4×10^5 cells/mL. Cell suspensions were aliquoted at 60- μ L/well into a 96-well plate. Various concentrations of rhTPO (R&D) and COS7 culture supernatants or purified samples were prepared, and a 40- μ L aliquot was added into each well. The plates were then incubated at 37°C under 5% CO₂ for 24 hr. Immediately after a 10- μ L aliquot of WST-8 reagent (Cell Count Reagent SF; Nacalai Tesque) was added into each well, absorbance was measured at 450 nm (and at 655 nm as a control) using Benchmark Plus. After two hours of incubation, absorbance was again measured at 450 nm (and at 655 nm as a control). The WST-8 reagent changes colors at 450 nm in a color reaction that reflects the viable cell count. The TPO-like

agonistic activity was assessed using the change in absorbance during the two-hour incubation as an index. EC₅₀ values were computed using GraphPad Prism.

[0138]

TPO-like agonistic activity was assayed using the human leukemia cell line M-07e (purchased from DSMZ) which proliferates TPO-dependently. M-07e cells were washed twice with RPMI 1640/1% FBS, and then suspended in RPMI 1640/10% FBS to a concentration of 5×10^5 cells/mL. The resulting cell suspension was aliquoted at 50- μ L/well into a 96-well plate. Various concentrations of rhTPO and COS7 culture supernatants or purified samples were prepared, and a 50- μ L aliquot was added into each well. The plates were then incubated at 37°C under 5% CO₂ for 48 hr. Immediately after a 10- μ L aliquot of WST-8 reagent (Cell Count Reagent SF; Nacalai Tesque) was added to each well, absorbance of was measured at 450 nm (and at 655 nm as a control) using a Benchmark Plus. After four hours of incubation, absorbance was again measured at 450 nm (and at 655 nm as a control). The TPO-like agonistic activity was assayed using the change in absorbance during the four-hour incubation as an index.

[0139]

Purified VB22B IgG, VB22B diabody, and VB22B sc(Fv)₂ were assayed for their TPO-like agonistic activities using BaF3-human Mpl, BaF3-monkey Mpl, and M-07e. The results are shown in Figures 3, 4, and 5, respectively. The presence of bivalent antigen-binding domains in a single antibody molecule is essential for its agonistic activity. The distance and angle between two antigen-binding domains can also be important factors (see WO 02/33073 and WO 02/33072). Similar results were obtained for the newly isolated anti-human Mpl antibodies. Specifically, the agonistic activities of VB22B diabody and VB22B sc(Fv)₂ (EC₅₀ = 61 pM and 27 pM in BaF-human Mpl, respectively) were higher than that of VB22B IgG (EC₅₀ > 30 nM in BaF-human Mpl), and were equivalent to or higher than that of the naturally-occurring human TPO ligand (EC₅₀ = 76 pM in BaF-human Mpl). The VB22B diabody activity was lower than that of VB22B sc(Fv)₂. This suggests that the structure of a single-chain antibody is greatly altered by its molecular shape and the length of the linker sequence, which in turn changes the agonistic activity. Sixteen types of the single-chain anti-human Mpl antibodies were obtained, each exhibiting a high agonistic activity. The amino acid sequences of the H chain and L chain variable regions of the representative antibodies are shown in Figures 6 and 7, respectively.

[0140]

2.9 Humanization of single-chain anti-human Mpl antibody

Antibody sequence data for the humanization of VB22B sc(Fv)₂ were obtained from the Kabat Database (<ftp://ftp.ebi.ac.uk/pub/databases/kabat/>), and homology searches were carried

out independently for the H chain variable region and the L chain variable region. As a result, the H chain variable region was found to be highly homologous to DN13 (Smithson S. L. *et al.*, Mol Immunol. (1999) 36: 113-124). The L chain variable region was found to be highly homologous to ToP027 (Hougs L. *et al.*, J. Immunol. (1999) 162: 224-237). Humanized antibodies were prepared by inserting a complementarity-determining region (hereinafter abbreviated as “CDR”) into the framework regions (hereinafter abbreviated as “FR”) of the above antibodies.

[0141]

Specifically, synthetic oligo-DNAs of approximately 50 nucleotides in length were designed as to make 20 of these nucleotides available for hybridization, and the synthetic oligo-DNAs were assembled by PCR to prepare genes that encode the respective variable regions. Using the resulting genes, sc(Fv)₂ was similarly prepared by the method described in Example 2.3. The respective DNAs were cloned into a pCXND3 expression vector to construct expression vectors pCXND3-hVB22B sc(Fv)₂. The nucleotide sequence and the amino acid sequence of hVB22B sc(Fv)₂ in plasmid are shown in SEQ ID NO:1 and SEQ ID NO: 2.

[0142]

[Example 3] Preparation of anti-Mpl diabodies by the AGS method

Anti-Mpl diabodies having agonistic activity were prepared by an Autocrine Growth Selection (AGS) method (see, WO 03/91424).

[0143]

3.1 Construction of a retrovirus library

Spleens were isolated from MRL/lpr mice immunized with shMPL-Flag by the method described in Example 1.5, and homogenized in TRIZOL Reagent (Invitrogen) using a Dounce homogenizer. After chloroform addition, the homogenized sample was shaken vigorously, the aqueous phase was removed and total RNA was extracted by isopropanol precipitation. mRNA was purified using a PolyA Tract System 1000 (Promega). Reverse transcription of 2.5 µg mRNA was carried out at 42°C for 50 min using the Superscript First strand synthesis system for RT-PCR (Invitrogen) and the included oligo-dT primers to prepare cDNA.

[0144]

The composition of the PCR reaction solution (250 µL) is shown below.

10x KOD Plus Buffer (Toyobo)	25 µL
2 mM dNTPs (dATP, dGTP, dCTP, and dTTP) (Toyobo)	25 µL
2.5 mM MgSO ₄ (Toyobo)	10 µL
KOD Plus (Toyobo)	7.5 µL
Reverse transcription products	25 µL
Mixed primers complementary to H chain or L chain variable region	500 pmol

[0145]

The reaction conditions were:

98°C (initial temperature) for 3 min;

32 cycles of 98°C for 20 sec, 58°C for 20 sec, and 72°C for 30 sec;

and final extension was at 72°C for 6 min.

[0146]

The H chain primer mix contained HS1 to HS19 (SEQ ID NOs: 178 to 196) and HA1 to HA4 (SEQ ID NOs: 197 to 200), which were mixed at the indicated ratios next to the sequence names in Table 1. The L chain primer mix contained LS1 to LS17 (SEQ ID NOs: 201 to 217), LSlambda (SEQ ID NO: 218), LA1 to LA5 (SEQ ID NOs: 219 to 222), and LAlambda (SEQ ID NO: 223). The respective PCR products were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN). The H chain and L chain variable regions were linked via the (Gly₄Ser)₁ linker sequence by PCR using sc-S (SEQ ID NO: 224) and sc-AS (SEQ ID NO: 225) as described below.

[0147]

The composition of the PCR reaction solution (100 µL in total) is shown below.

10x KOD Plus Buffer (Toyobo)	10 µL
2 mM dNTPs (dATP, dGTP, dCTP, and dTTP) (Toyobo)	10 µL
2.5 mM MgSO ₄ (Toyobo)	4 µL
KOD Plus (Toyobo)	2 µL
Fragment of H chain variable region	4 µL
Fragment of L chain variable region	4 µL

The first-round PCR conditions were:

94°C (initial temperature) for 3 min; and

seven cycles of 94°C for 1 min and 63°C for 4 min.

Then, sc-S and sc-AS (25 pmol each) were added to the first-round products.

[0148]

The second-round PCR conditions were:

30 cycles of 94°C for 30 sec, 55°C for 2 min, and 72°C for 2 min;

and final extension was at 72°C for 6 min.

[0149]

The resulting product with an *Sfi*I restriction site at both ends was purified using the QIAquick PCR Purification Kit (QIAGEN), and incubated with the *Sfi*I restriction enzyme (TaKaRa) overnight at 50°C. The PCR product purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN) was inserted into the *Sfi*I site of the viral vector pMX/IL3ssGFPHis.

[0150]

The resulting plasmid was constructed by inserting a GFP gene, which has an *EcoRI* site, mouse IL-3 signal sequence and *SfiI* site at its 5' end; and an *SfiI* site, His tag sequence, termination codon, and *NotI* site at its 3' end, between the *EcoRI* and *NotI* sites on the pMX viral
5 vector (Onishi, M. *et al.*, Mol. Cell. Biol. 18: 3871-3879). The plasmid was introduced into the ElectroMAX DH10B T1 phage resistant cells (Invitrogen) by electroporation (settings: 2.5 kV, 25 μ F, and 100 Ω) using a Gene Pulser II (Bio-Rad). The cells were plated onto an LB-Agar plate containing 100 μ g/mL ampicillin. After overnight incubation, 1×10^7 colonies were obtained. Colonies were recovered from the plate and plasmids were then extracted using the
10 QIAGEN Plasmid Maxi Kit (QIAGEN).

[0151]

[Table 1]

SEQ ID NO:178 (HS1(4))	GCCCAGCCGGCCATGGCGGAKGTRMAGCTTCAGGAGTC
SEQ ID NO:179 (HS2(4))	GCCCAGCCGGCCATGGCGGAGGTBCAGCTBCAGCAGTC
SEQ ID NO:180 (HS3(3))	GCCCAGCCGGCCATGGCGCAGGTGCAGCTGAAGSASTC
SEQ ID NO:181 (HS4(4))	GCCCAGCCGGCCATGGCGGAGGTCCARCTGCAACARTC
SEQ ID NO:182 (HS5(7))	GCCCAGCCGGCCATGGCGCAGGTYCAGCTBCAGCARTC
SEQ ID NO:183 (HS6(2))	GCCCAGCCGGCCATGGCGCAGGTYCARCTGCAGCAGTC
SEQ ID NO:184 (HS7(1))	GCCCAGCCGGCCATGGCGCAGGTCCACGTGAAGCAGTC
SEQ ID NO:185 (HS8(2))	GCCCAGCCGGCCATGGCGGAGGTGAASSTGGTGGAAATC
SEQ ID NO:186 (HS9(5))	GCCCAGCCGGCCATGGCGGAVGTGAWGYTGGTGGAGTC
SEQ ID NO:187 (HS10(2))	GCCCAGCCGGCCATGGCGGAGGTGCAGSKGGTGGAGTC
SEQ ID NO:188 (HS11(2))	GCCCAGCCGGCCATGGCGGAKGTGCAMCTGGTGGAGTC
SEQ ID NO:189 (HS12(2))	GCCCAGCCGGCCATGGCGGAGGTGAAGCTGATGGARTC
SEQ ID NO:190 (HS13(1))	GCCCAGCCGGCCATGGCGGAGGTGCARCTTGTGAGTC
SEQ ID NO:191 (HS14(2))	GCCCAGCCGGCCATGGCGGARGTRAAGCTTCTCGAGTC
SEQ ID NO:192 (HS15(2))	GCCCAGCCGGCCATGGCGGAAGTGAARSTTGAGGAGTC
SEQ ID NO:193 (HS16(5))	GCCCAGCCGGCCATGGCGCAGGTTACTCTRAAAGWGTSTG
SEQ ID NO:194 (HS17(3.5))	GCCCAGCCGGCCATGGCGCAGGTCCAAC TVCAGCARCC
SEQ ID NO:195 (HS18(0.7))	GCCCAGCCGGCCATGGCGGATGTGAACTTGGAAGTGTC
SEQ ID NO:196 (HS19(0.7))	GCCCAGCCGGCCATGGCGGAGGTGAAGGTCATCGAGTC
SEQ ID NO:197 (HA1(1))	GGAGCCGCCGCCGCCGCCGAGGAAACGGTGACCGTGGT
SEQ ID NO:198 (HA2(1))	GGAGCCGCCGCCGCCGCCGAGGAGACTGTGAGAGTGGT
SEQ ID NO:199 (HA3(1))	GGAGCCGCCGCCGCCGCCGAGAGACAGTGACCAGAGT
SEQ ID NO:200 (HA4(1))	GGAGCCGCCGCCGCCGCCGAGGAGACGGTGACTGAGGT
SEQ ID NO:201 (LS1(1))	GGCGGCGGCGGCTCCGAYATCCAGCTGACTCAGCC
SEQ ID NO:202 (LS2(2))	GGCGGCGGCGGCTCCGAYATTGTTCTCWCCAGTC
SEQ ID NO:203 (LS3(5))	GGCGGCGGCGGCTCCGAYATTGTGMTMACTCAGTC
SEQ ID NO:204 (LS4(3.5))	GGCGGCGGCGGCTCCGAYATTGTGYTRACACAGTC
SEQ ID NO:205 (LS5(4))	GGCGGCGGCGGCTCCGAYATTGTRATGACMCAGTC
SEQ ID NO:206 (LS6(7))	GGCGGCGGCGGCTCCGAYATTMAGATRAMCCAGTC
SEQ ID NO:207 (LS7(6))	GGCGGCGGCGGCTCCGAYATTCAGATGAYDCAGTC
SEQ ID NO:208 (LS8(1.5))	GGCGGCGGCGGCTCCGAYATYCAGATGACACAGAC
SEQ ID NO:209 (LS9(2))	GGCGGCGGCGGCTCCGAYATTGTTCTCAWCCAGTC
SEQ ID NO:210 (LS10(3.5))	GGCGGCGGCGGCTCCGAYATTGWGCTSACCCAATC
SEQ ID NO:211 (LS11(8))	GGCGGCGGCGGCTCCGAYATTSTRATGACCCARTC
SEQ ID NO:212 (LS12(8))	GGCGGCGGCGGCTCCGAYRTTKTGATGACCCARAC
SEQ ID NO:213 (LS13(6))	GGCGGCGGCGGCTCCGAYATTGTGATGACBCAGKC
SEQ ID NO:214 (LS14(2))	GGCGGCGGCGGCTCCGAYATTGTGATAACYCAGGA
SEQ ID NO:215 (LS15(2))	GGCGGCGGCGGCTCCGAYATTGTGATGACCCAGWT
SEQ ID NO:216 (LS16(1))	GGCGGCGGCGGCTCCGAYATTGTGATGACACAACC
SEQ ID NO:217 (LS17(1))	GGCGGCGGCGGCTCCGAYATTTTGCTGACTCAGTC
SEQ ID NO:218 (LS1lambda(1))	GGCGGCGGCGGCTCCGATGCTGTTGTGACTCAGGAATC
SEQ ID NO:219 (LA1(4))	GGAATTCGGCCCCCGAGGCCTTGATTTCCAGCTTGG
SEQ ID NO:220 (LA2(4))	GGAATTCGGCCCCCGAGGCCTTTATTTCCAGCTTGG
SEQ ID NO:221 (LA4(4))	GGAATTCGGCCCCCGAGGCCTTTATTTCCAACCTTG
SEQ ID NO:222 (LA5(4))	GGAATTCGGCCCCCGAGGCCTTCAGCTCCAGCTTGG
SEQ ID NO:223 (LA1lambda(1))	GGAATTCGGCCCCCGAGGCCCTAGGACAGTCAGTTTGG

[0152]

3.2 Establishment of autonomously replicating cell lines by the AGS method

The resulting library was transfected into a packaging cell, Pt-E, (Morita, S. *et al.*, Gene therapy 7: 1063-1066) using FuGENE 6 (Roche Diagnostics). Specifically, Pt-E was plated onto 6-cm dishes and cultured in DMEM/10% FBS (Invitrogen). A mixture of FuGENE 6 and the library was added to the plate the following day. The culture medium was exchanged the next day, and the culture supernatant was collected 24 hours after that. 10 µg/mL polybrene (Hexadimethrine Bromide; Sigma) and 2 ng/mL mIL-3 were added to the culture supernatant containing recombinant virus particles. The viral solution was used to infect the BaF-monkey Mpl target cells. The cells were washed with PBS the following day, and suspended in RPMI 1640/10% FBS without mIL-3. The suspension was plated onto a 96-well plate at a cell density of 1,000 cells/well. Autonomously replicating cell lines (AB317 and AB324) were obtained after seven days of incubation. Genomic DNAs were extracted from these cells using a DNeasy Tissue Kit (QIAGEN), and the antibody genes were amplified by PCR.

[0153]

The composition of the PCR reaction solution (50 µL in total) is shown below.

10x LA Taq Buffer (TaKaRa)	5 µL
2 mM dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	5 µL
2.5 mM MgCl ₄ (TaKaRa)	5 µL
TaKaRa LA Taq (TaKaRa)	0.5 µL
Genomic DNA	0.5 µg
AGSdbS1 (SEQ ID NO: 226) and AGSdbA1 (SEQ ID NO: 227)	25 pmol

[0154]

The reaction conditions were:

94°C (initial temperature) for 1 min;

30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 70°C for 1 min;

and final extension was at 72°C for 6 min.

[0155]

The nucleotide sequence and the amino acid sequence of the H chain of cloned AB317 are shown in SEQ ID NOs: 154 and 155. The nucleotide sequence and the amino acid sequence of AB317 L chain are shown in SEQ ID NOs: 156 and 157. The nucleotide sequence and the amino acid sequence of AB324 H chain are shown in SEQ ID NOs: 158 and 159. The nucleotide sequence and the amino acid sequence of AB324 L chain are shown in SEQ ID NOs: 160 and 161.

[0156]

3.3 Activity assays of the diabodies obtained by AGS method

Each of the anti-Mpl diabodies obtained above was inserted into the pCXND3 expression vector. The PCR primers used are a synthetic oligonucleotide complementary to the 5' end of the diabody and containing an *EcoRI* site, and a synthetic oligonucleotide complementary to the nucleotide sequence of the 3' end of the diabody and containing a FLAG tag and a *NotI* site. The PCR product thus obtained was inserted into pCXND3 between the *EcoRI* and *NotI* sites. The diabody was expressed transiently in COS7 cells by the method described in Example 2.4. The culture supernatant was removed and the activity of the diabody was evaluated.

[0157]

The binding activities of the diabodies were assessed by flow cytometry using CHO cells that express Mpl derived from various species (Fig. 8). AB317 was proven to bind to CHO-mouse Mpl.

[0158]

The TPO-like agonistic activities of the diabodies were evaluated using BaF-human Mpl, BaF-monkey Mpl, and BaF-mouse Mpl (Figures 9, 10, and 11). AB317 had the highest agonistic activity against human, monkey, and mouse Mpl, whereas AB324 showed the highest agonistic activity against human and monkey Mpl.

[0159]

This proves that anti-Mpl diabodies having high agonistic activity can be obtained by the AGS method.

[Brief Description of the Drawings]

[0160]

[Fig. 1] Fig. 1 demonstrates the strategy for preparing single-chain antibody sc(Fv)₂.

[Fig. 2] Fig. 2 illustrates the assessment of VB22B sc(Fv)₂ binding activity using an Mpl-expressing CHO cell line. Purified VB22B sc(Fv)₂ was used.

[Fig. 3] Fig. 3 illustrates the assessment of VB22B antibody agonistic activity using BaF-human Mpl.

[Fig. 4] Fig. 4 illustrates the assessment of VB22B antibody agonistic activity using BaF-monkey Mpl.

[Fig. 5] Fig. 5 illustrates the assessment of VB22B antibody agonistic activity using M-07e.

[Fig. 6] Fig. 6 shows the amino acid sequences of anti-human Mpl antibodies (H chains) that exhibit higher agonistic activities when converted into minibodies.

[Fig. 7] Fig. 7 shows the amino acid sequences of anti-human Mpl antibodies (L chains) which exhibit higher agonistic activities when converted into minibodies.

[Fig. 8] Fig. 8 illustrates the binding activity assessment of AB317 diabody using

Mpl-expressing CHO cells. Both VB22B diabody (solid line) and AB317 diabody (broken line) were obtained from COS7 culture supernatants.

[Fig. 9] Fig. 9 illustrates the agnostic activity assessment of AB324 and AB317 diabodies using BaF-human Mpl.

5 [Fig. 10] Fig. 10 illustrates the agnostic activity assessment of AB324 and AB317 diabodies using BaF-monkey Mpl.

[Fig. 11] Fig. 11 illustrates the agnostic activity assessment of AB324 and AB317 diabodies using BaF-mouse Mpl.

[Sequence Listing]

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Arg Met Ser Asn Leu Ala Ser
1 5

10 <210> 92
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15 <400> 92
Met Gln His Leu Glu Tyr Pro Tyr Thr
1 5

20 <210> 93
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25 <400> 93
Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr
1 5 10 15

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Arg Met Ser Asn Leu Ala Ser

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1 5

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20 Arg Ser Ser Lys Ser Leu Leu Tyr Ser Asn Gly Asn Thr Tyr Leu Tyr

1 5 10 15

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30 Arg Met Ser Asn Leu Ala Ser

1 5

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Met Gln His Leu Glu Tyr Pro Tyr Thr

5 1 5

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<211> 16

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<400> 99

Arg Ser Ser Lys Ser Leu Leu Tyr Ser Asn Gly Asn Ile Tyr Leu Tyr

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Arg Met Ser Asn Leu Ala Ser

25 1 5

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Met Gln His Leu Glu Tyr Pro Tyr Thr

35 1 5

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Arg Ser Ser Lys Ser Leu Leu His Asn Asn Gly Asn Thr Tyr Leu Tyr

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Arg Met Ser Asn Leu Ala Ser

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Met Gln His Ile Glu Tyr Pro Phe Thr

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Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr

1 5 10 15

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Arg Met Ser Asn Leu Ala Ser

1 5

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Met Gln His Leu Glu Tyr Pro Tyr Thr

1 5

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Arg Ala Ser Glu Ser Val Glu Tyr Tyr Gly Thr Ser Leu Met Gln

1 5 10 15

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Gly Ala Ser Asn Val Glu Ser
1 5

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Gln Gln Ser Arg Lys Val Pro Trp Thr
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Lys Ala Ser Gln Asn Val Gly Asn Ile Ile Ala
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Leu Ala Ser Tyr Arg Tyr Ser

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10 Gln Gln Tyr Ser Ser Ser Pro Leu Thr

1 5

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20 Ser Ala Ser Ser Ser Val Ser Ser Ser His Leu Tyr

1 5 10

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25 <211> 7

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30 Ser Thr Ser Asn Leu Ala Ser

1 5

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35 <211> 9

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<400> 116

His Gln Trp Ser Ser Tyr Pro Trp Thr

5 1 5

<210> 117

<211> 411

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<220>

<221> CDS

15 <222> (1)..(411)

<223>

<400> 117

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20 Met Glu Trp Pro Leu Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1 5 10 15

gtc cac tcc cag gtt cag ctg cag cag tct gga cct gag ctg gtg aag 96
Val His Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
25 20 25 30

cct ggg gcc tca gtg aag att tcc tgc aag gct tct ggc tat gca ttc 144
Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe
35 40 45

act aac tcc tgg atg aac tgg gtg aag cag agg cct gga aag ggt ctt 192
Thr Asn Ser Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu
50 55 60

gag tgg att gga cgg att tat cct gga gat gga gaa act atc tac aat 240
Glu Trp Ile Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Ile Tyr Asn

	65	70	75	80	
	ggg aaa ttc agg gtc aag gcc aca ctg act gca gac aaa tcc tcc agc				288
	Gly Lys Phe Arg Val Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser				
5		85	90	95	
	aca gcc tac atg gat atc agc agc ctg aca tct gag gac tct gcg gtc				336
	Thr Ala Tyr Met Asp Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val				
		100	105	110	
10					
	tac ttc tgt gca aga ggc tat gat gat tac tcg ttt gct tac tgg ggc				384
	Tyr Phe Cys Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly				
		115	120	125	
15	caa ggg act ctg gtc act gtc tct gca				411
	Gln Gly Thr Leu Val Thr Val Ser Ala				
		130	135		
20	<210> 118				
	<211> 137				
	<212> PRT				
	<213> Mus musculus				
25	<400> 118				
	Met Glu Trp Pro Leu Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly				
	1	5	10	15	
	Val His Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys				
30		20	25	30	
	Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe				
		35	40	45	
35	Thr Asn Ser Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu				
	50	55	60		

Glu Trp Ile Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Ile Tyr Asn
65 70 75 80

5 Gly Lys Phe Arg Val Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
85 90 95

Thr Ala Tyr Met Asp Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
100 105 110

10 Tyr Phe Cys Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly
115 120 125

Gln Gly Thr Leu Val Thr Val Ser Ala
15 130 135

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<211> 396
20 <212> DNA
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<220>
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25 <222> (1).. (396)
<223>

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1 5 10 15

gga gcc att ggg gat att gtg atg act cag gct gca ccc tct ata cct 96
Gly Ala Ile Gly Asp Ile Val Met Thr Gln Ala Ala Pro Ser Ile Pro
35 20 25 30

	gtc act cct gga gag tca gta tcc atc tcc tgt agg tct agt aag agt	144
	Val Thr Pro Gly Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser	
	35 40 45	
5	ctc ctg cat agt aat ggc aac act tac ttg tat tgg ttc ctg cag agg	192
	Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg	
	50 55 60	
10	cca ggc cag tct cct caa ctc ctg ata tat cgg atg tcc aac ctt gcc	240
	Pro Gly Gln Ser Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala	
	65 70 75 80	
15	tca gga gtc cca gat agg ttc agt ggc agt ggg tca gga act gct ttc	288
	Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe	
	85 90 95	
20	aca ctg aga atc agt aga gtg gag gct gag gat gtg ggt gtt tat tac	336
	Thr Leu Arg Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr	
	100 105 110	
	tgt atg caa cat ata gaa tat cct ttt acg ttc gga tcg ggg acc aag	384
	Cys Met Gln His Ile Glu Tyr Pro Phe Thr Phe Gly Ser Gly Thr Lys	
	115 120 125	
25	ctg gaa ata aaa	396
	Leu Glu Ile Lys	
	130	
30	<210> 120	
	<211> 132	
	<212> PRT	
	<213> Mus musculus	
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	Met Arg Cys Leu Ala Glu Phe Leu Gly Leu Leu Val Phe Trp Ile Pro	

	1	5	10	15	
	Gly	Ala	Ile	Gly	Asp
			Ile	Val	Met
			Thr	Gln	Ala
			Ala	Pro	Ser
			Ile	Pro	
		20	25	30	
5	Val	Thr	Pro	Gly	Glu
			Ser	Val	Ser
			Ile	Ser	Cys
			Arg	Ser	Ser
			Lys	Ser	
		35	40	45	
	Leu	Leu	His	Ser	Asn
			Gly	Asn	Thr
			Tyr	Leu	Tyr
			Trp	Phe	Leu
			Gln	Arg	
10	50	55	60		
	Pro	Gly	Gln	Ser	Pro
			Gln	Leu	Leu
			Ile	Tyr	Arg
			Met	Ser	Asn
			Leu	Ala	
	65	70	75	80	
	Ser	Gly	Val	Pro	Asp
			Arg	Phe	Ser
			Gly	Ser	Gly
			Ser	Gly	Thr
			Ala	Phe	
		85	90	95	
	Thr	Leu	Arg	Ile	Ser
			Arg	Val	Glu
			Ala	Glu	Asp
			Val	Gly	Val
			Tyr	Tyr	
		100	105	110	
20	Cys	Met	Gln	His	Ile
			Glu	Tyr	Pro
			Phe	Thr	Phe
			Gly	Ser	Gly
			Thr	Lys	
		115	120	125	
	Leu	Glu	Ile	Lys	
25	130				
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	<211>	762			
30	<212>	DNA			
	<213>	Mus musculus			
	<400>	121			
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		ccactcccag			60
35	gttcagctgc	agcagtctgg	acctgagctg	gtgaagcctg	gggcctcagt
		gaagatttcc			120

tgcaaggctt ctggctatgc attcactaac tcctggatga actgggtgaa gcagaggcct 180
 ggaaagggtc ttgagtggat tggacggatt tatcctggag atggagaaac tatctacaat 240
 5 gggaattca gggtaaggc cacactgact gcagacaaat cctccagcac agcctacatg 300
 gatatcagca gcctgacatc tgaggactct gcggtctact tctgtgcaag aggctatgat 360
 10 gattactcgt ttgcttactg gggccaaggg actctggatca ctgtctctgc aggtggtggt 420
 ggttcggata ttgtgatgac tcaggctgca ccctctatac ctgtcactcc tggagagtca 480
 gtatccatct cctgtaggtc tagtaagagt ctctgcata gtaatggcaa cacttacttg 540
 15 tattggttcc tgcagaggcc aggccagtct cctcaactcc tgatatatcg gatgtccaac 600
 cttgcctcag gagtcccaga taggttcagt ggcagtgggt caggaactgc ttccacactg 660
 20 agaatcagta gagtggaggc tgaggatgtg ggtgtttatt actgtatgca acatatagaa 720
 tatcctttta cgttcggatc ggggaccaag ctggaaataa aa 762

25 <210> 122
 <211> 254
 <212> PRT
 <213> Mus musculus

30 <400> 122
 Met Glu Trp Pro Leu Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
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 Val His Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
 35 20 25 30

	Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe
	35 40 45
5	Thr Asn Ser Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu
	50 55 60
	Glu Trp Ile Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Ile Tyr Asn
	65 70 75 80
10	Gly Lys Phe Arg Val Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
	85 90 95
	Thr Ala Tyr Met Asp Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
15	100 105 110
	Tyr Phe Cys Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly
	115 120 125
	Gln Gly Thr Leu Val Thr Val Ser Ala Gly Gly Gly Gly Ser Asp Ile
20	130 135 140
	Val Met Thr Gln Ala Ala Pro Ser Ile Pro Val Thr Pro Gly Glu Ser
	145 150 155 160
25	Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly
	165 170 175
	Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser Pro Gln
30	180 185 190
	Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro Asp Arg
	195 200 205
	Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile Ser Arg
35	210 215 220

Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His Ile Glu
 225 230 235 240

5 Tyr Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
 245 250

<210> 123

<211> 635

10 <212> PRT

<213> Homo sapiens

<400> 123

15 Met Pro Ser Trp Ala Leu Phe Met Val Thr Ser Cys Leu Leu Leu Ala
 1 5 10 15

Pro Gln Asn Leu Ala Gln Val Ser Ser Gln Asp Val Ser Leu Leu Ala
 20 25 30

20 Ser Asp Ser Glu Pro Leu Lys Cys Phe Ser Arg Thr Phe Glu Asp Leu
 35 40 45

Thr Cys Phe Trp Asp Glu Glu Glu Ala Ala Pro Ser Gly Thr Tyr Gln
 50 55 60

25 Leu Leu Tyr Ala Tyr Pro Arg Glu Lys Pro Arg Ala Cys Pro Leu Ser
 65 70 75 80

30 Ser Gln Ser Met Pro His Phe Gly Thr Arg Tyr Val Cys Gln Phe Pro
 85 90 95

Asp Gln Glu Glu Val Arg Leu Phe Phe Pro Leu His Leu Trp Val Lys
 100 105 110

35 Asn Val Phe Leu Asn Gln Thr Arg Thr Gln Arg Val Leu Phe Val Asp
 115 120 125

Ser Val Gly Leu Pro Ala Pro Pro Ser Ile Ile Lys Ala Met Gly Gly
130 135 140

5 Ser Gln Pro Gly Glu Leu Gln Ile Ser Trp Glu Glu Pro Ala Pro Glu
145 150 155 160

Ile Ser Asp Phe Leu Arg Tyr Glu Leu Arg Tyr Gly Pro Arg Asp Pro
165 170 175

10 Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala Thr Glu Thr
180 185 190

Cys Cys Pro Ala Leu Gln Arg Pro His Ser Ala Ser Ala Leu Asp Gln
15 195 200 205

Ser Pro Cys Ala Gln Pro Thr Met Pro Trp Gln Asp Gly Pro Lys Gln
210 215 220

20 Thr Ser Pro Ser Arg Glu Ala Ser Ala Leu Thr Ala Glu Gly Gly Ser
225 230 235 240

Cys Leu Ile Ser Gly Leu Gln Pro Gly Asn Ser Tyr Trp Leu Gln Leu
245 250 255

25 Arg Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp Gly Ser Trp
260 265 270

Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val Ala Leu Gly
30 275 280 285

Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys Gln Trp Gln
290 295 300

35 Gln Gln Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His Ser Arg Ala
305 310 315 320

Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asn Cys Glu Glu
325 330 335

5 Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe Ser Arg Cys
340 345 350

His Phe Lys Ser Arg Asn Asp Ser Ile Ile His Ile Leu Val Glu Val
355 360 365

10 Thr Thr Ala Pro Gly Thr Val His Ser Tyr Leu Gly Ser Pro Phe Trp
370 375 380

Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His Trp Arg Glu
15 385 390 395 400

Ile Ser Ser Gly His Leu Glu Leu Glu Trp Gln His Pro Ser Ser Trp
405 410 415

20 Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly Glu Gly His
420 425 430

Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg Gly Gly Thr
435 440 445

25 Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu Arg Ala Arg
450 455 460

Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp Ser Asp Pro
30 465 470 475 480

Thr Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser Leu Val Thr
485 490 495

35 Ala Leu His Leu Val Leu Gly Leu Ser Ala Val Leu Gly Leu Leu Leu
500 505 510

Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg His Ala Leu
515 520 525

5 Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln Tyr Leu Arg
530 535 540

Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser Asp Thr Cys
545 550 555 560

10 Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys Ser Ser Glu
565 570 575

Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ala Gln Met Asp Tyr Arg
15 580 585 590

Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser Val Cys Pro
595 600 605

20 Pro Met Ala Glu Ser Gly Ser Cys Cys Thr Thr His Ile Ala Asn His
610 615 620

Ser Tyr Leu Pro Leu Ser Tyr Trp Gln Gln Pro
625 630 635

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<210> 124
<211> 122
<212> PRT

30 <213> Mus musculus

<400> 124
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1 5 10 15

35 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser

20 25 30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
35 40 45

5
Gly Arg Thr Tyr Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe
50 55 60

10
Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
65 70 75 80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
85 90 95

15
Ala Arg Gly Trp Ile Leu Ala Asp Gly Gly Tyr Ser Phe Ala Tyr Trp
100 105 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ala
115 120

20

<210> 125
<211> 112
<212> PRT
25 <213> Mus musculus

<400> 125
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Ile Pro Val Thr Pro Gly
1 5 10 15

30
Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
35 35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
 50 55 60

5 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
 65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Ile Tyr Tyr Cys Met Gln His
 85 90 95

10 Leu Glu Tyr Pro Phe Thr Phe Gly Thr Gly Thr Lys Leu Glu Ile Lys
 100 105 110

<210> 126
 15 <211> 118
 <212> PRT
 <213> Mus musculus

<400> 126
 20 Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
 1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser
 20 25 30

25 Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
 35 40 45

Gly Arg Ile Tyr Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe
 30 50 55 60

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
 65 70 75 80

35 Ile Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
 85 90 95

Ala Arg Gly Tyr Ala Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
100 105 110

5 Leu Val Thr Val Ser Ala
115

<210> 127
10 <211> 112
<212> PRT
<213> Mus musculus

<400> 127
15 Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1 5 10 15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
20 25 30

20 Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
25 50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
65 70 75 80

30 Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
85 90 95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
100 105 110

35

<210> 128

<211> 118

<212> PRT

<213> Mus musculus

5

<400> 128

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1 5 10 15

10 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser
20 25 30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
35 40 45

15

Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Tyr Asn Gly Lys Phe
50 55 60

20 Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Asn Thr Ala Tyr
65 70 75 80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
85 90 95

25 Ala Arg Gly Phe Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
100 105 110

Leu Val Thr Val Ser Ala
115

30

<210> 129

<211> 112

<212> PRT

35 <213> Mus musculus

<400> 129

Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1 5 10 15

5 Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
35 40 45

10

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Ala Pro
50 55 60

15 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
65 70 75 80

Ser Arg Val Glu Thr Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
85 90 95

20 Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
100 105 110

<210> 130

25 <211> 118

<212> PRT

<213> Mus musculus

<400> 130

30 Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Ser Ser Ser
20 25 30

35

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile

35 40 45

Gly Arg Ile Tyr Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe
50 55 60

5

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
65 70 75 80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
10 85 90 95

Ala Ser Gly Tyr Ala Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
100 105 110

15 Leu Val Thr Val Ser Ala
115

<210> 131

20 <211> 112
<212> PRT
<213> Mus musculus

<400> 131

25 Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1 5 10 15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
20 25 30

30 Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
35 50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
5 85 90 95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
100 105 110

10
<210> 132
<211> 118
<212> PRT
<213> Mus musculus

15
<400> 132
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1 5 10 15

20 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Arg Ser
20 25 30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
35 40 45

25 Gly Arg Ile Tyr Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe
50 55 60

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
30 65 70 75 80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
85 90 95

35 Ala Ser Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
100 105 110

Leu Val Thr Val Ser Ala

115

5

<210> 133

<211> 112

<212> PRT

<213> Mus musculus

10

<400> 133

Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly

1 5 10 15

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Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser

20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser

35 40 45

20

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro

50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile

25 65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His

85 90 95

30

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys

100 105 110

<210> 134

35 <211> 118

<212> PRT

<213> Mus musculus

<400> 134

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
5 1 5 10 15
Ser Val Lys Ile Ser Cys Arg Ala Phe Gly Tyr Ala Phe Ser Asn Ser
20 25 30
10 Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
35 40 45
Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Asn Asn Gly Lys Phe
50 55 60
15 Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
65 70 75 80
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
20 85 90 95
Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
100 105 110
25 Leu Val Thr Val Ser Ala
115

<210> 135

30 <211> 112

<212> PRT

<213> Mus musculus

<400> 135

35 Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1 5 10 15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
20 25 30

5 Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
50 55 60

10 Asp Arg Phe Ser Gly Ser Gly Ser Gly Ala Ala Phe Thr Leu Arg Ile
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
15 85 90 95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
100 105 110

20

<210> 136
<211> 115
<212> PRT
<213> Mus musculus

25

<400> 136
Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys Pro Gly Ala
1 5 10 15

30 Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
20 25 30

Trp Val Asn Trp Val Lys Gln Arg Pro Gly Arg Gly Leu Glu Trp Ile
35 40 45

35 Gly Arg Ile His Pro Ser Asp Ser Glu Thr His Cys Asn Gln Lys Phe

50 55 60

Lys Arg Lys Ala Thr Leu Thr Val Asn Lys Ser Ser Ser Thr Ala Tyr
65 70 75 80

5

Ile Gln Leu His Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
85 90 95

Thr Ser Gly Gly Trp Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr
10 100 105 110

Val Ser Ala
115

15

<210> 137
<211> 112
<212> PRT
<213> Mus musculus

20

<400> 137
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1 5 10 15

25

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu Tyr Ser
20 25 30

Asn Gly Asn Ile Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
35 40 45

30

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
35 65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
 85 90 95

5 Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
 100 105 110

<210> 138
 <211> 118
 10 <212> PRT
 <213> Mus musculus

<400> 138
 Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
 15 1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser
 20 25 30

20 Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
 35 40 45

Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Asn Asn Gly Lys Phe
 50 55 60

25 Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Thr Thr Ala Tyr
 65 70 75 80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
 30 85 90 95

Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
 100 105 110

35 Leu Val Thr Val Ser Ala
 115

<210> 139

<211> 112

5 <212> PRT

<213> Mus musculus

<400> 139

10 Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1 5 10 15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
20 25 30

15 Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
50 55 60

20 Asp Arg Phe Ser Gly Ser Gly Ser Gly Ala Ala Phe Thr Leu Arg Ile
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
25 85 90 95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
100 105 110

30

<210> 140

<211> 118

<212> PRT

<213> Mus musculus

35

<400> 140

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
 1 5 10 15
 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Thr Ser
 5 20 25 30
 Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
 35 40 45
 10 Gly Arg Ile Tyr Pro Gly Asp Gly Glu Ala Asn Tyr Asn Gly Lys Phe
 50 55 60
 Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Ser Ala Tyr
 65 70 75 80
 15 Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
 85 90 95
 Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
 20 100 105 110
 Leu Val Thr Val Ser Ala
 115
 25
 <210> 141
 <211> 112
 <212> PRT
 <213> Mus musculus
 30
 <400> 141
 Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
 1 5 10 15
 35 Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
 20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Met Gln Arg Pro Gly Gln Ser
35 40 45

5 Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
65 70 75 80

10 Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
85 90 95

Val Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
15 100 105 110

<210> 142
<211> 118
20 <212> PRT
<213> Mus musculus

<400> 142
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
25 1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser
20 25 30

30 Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Pro Glu Trp Ile
35 40 45

Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Tyr Asn Gly Lys Phe
50 55 60

35 Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Val Tyr

65 70 75 80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
 85 90 95

5
Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
 100 105 110

Leu Val Thr Val Ser Ala
10 115

<210> 143
<211> 112
15 <212> PRT
<213> Mus musculus

<400> 143
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
20 1 5 10 15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
 20 25 30

25 Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
 35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
 50 55 60

30
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
35 85 90 95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
 100 105 110

5 <210> 144
 <211> 118
 <212> PRT
 <213> Mus musculus

10 <400> 144
 Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Leu Asn Pro Gly Ala
 1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Arg Ser
 15 20 25 30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
 35 40 45

20 Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Tyr Asn Gly Lys Phe
 50 55 60

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Thr Thr Ala Tyr
 65 70 75 80

25 Met Gln Phe Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
 85 90 95

Ala Arg Gly Asp Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
 30 100 105 110

Leu Val Thr Val Ser Ala
 115

35 <210> 145

<211> 112

<212> PRT

<213> Mus musculus

5 <400> 145

Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1 5 10 15

10 Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
35 40 45

15 Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
50 55 60

20 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
85 90 95

25 Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
100 105 110

<210> 146

<211> 115

30 <212> PRT

<213> Mus musculus

<400> 146

35 Gln Val Gln Leu Gln Gln Pro Gly Thr Glu Leu Val Arg Pro Gly Ala
1 5 10 15

Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Tyr
 20 25 30

Trp Val Asn Trp Val Lys Gln Arg Pro Gly Arg Gly Leu Glu Trp Ile
 5 35 40 45

Gly Arg Ile His Pro Tyr Asp Ser Glu Thr His Tyr Asn Gln Lys Phe
 50 55 60

10 Lys Asn Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr
 65 70 75 80

Ile Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
 85 90 95

15 Ala Ser Gly Gly Trp Phe Ala Ser Trp Gly Gln Gly Thr Leu Val Thr
 100 105 110

Val Ser Ala
 20 115

<210> 147
 <211> 112
 25 <212> PRT
 <213> Mus musculus

<400> 147
 Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
 30 1 5 10 15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu Tyr Ser
 20 25 30

35 Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
 35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
50 55 60

5 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Thr Ile
65 70 75 80

Ser Ser Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
85 90 95

10 Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
100 105 110

15 <210> 148
<211> 115
<212> PRT
<213> Mus musculus

20 <400> 148
Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys Pro Gly Ala
1 5 10 15

25 Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Tyr
20 25 30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Arg Gly Leu Glu Trp Ile
35 40 45

30 Gly Arg Ile His Pro Phe Asp Ser Glu Thr His Cys Ser Gln Lys Phe
50 55 60

Lys Asn Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Asn Thr Ala Tyr
65 70 75 80

35 Ile Gln Phe Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys

	85	90	95
	Ser Ser Gly Gly Trp Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr		
	100	105	110
5	Val Ser Ala		
	115		
10	<210> 149		
	<211> 112		
	<212> PRT		
	<213> Mus musculus		
15	<400> 149		
	Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Ser Val Thr Pro Gly		
	1 5 10 15		
	Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu Tyr Ser		
20	20 25 30		
	Asn Gly Asn Ile Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser		
	35 40 45		
25	Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro		
	50 55 60		
	Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Lys Ile		
	65 70 75 80		
30	Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His		
	85 90 95		
	Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys		
35	100 105 110		

<210> 150

<211> 118

<212> PRT

5 <213> Mus musculus

<400> 150

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala

1 5 10 15

10

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Thr Asn Ser

20 25 30

Trp Met Asn Trp Val Arg Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile

15 35 40 45

Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Ile Tyr Asn Gly Lys Phe

50 55 60

20 Arg Val Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr

65 70 75 80

Met Glu Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys

85 90 95

25

Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr

100 105 110

Leu Val Thr Val Ser Ala

30 115

<210> 151

<211> 112

35 <212> PRT

<213> Mus musculus

<400> 151

Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly

1 5 10 15

5

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Asn

20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser

10 35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro

50 55 60

15 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile

65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His

85 90 95

20

Ile Glu Tyr Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys

100 105 110

25 <210> 152

<211> 118

<212> PRT

<213> Mus musculus

30 <400> 152

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala

1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Asn Ser

35 20 25 30

Trp Met Asn Trp Val Asn Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
35 40 45

5 Gly Arg Ile Tyr Pro Gly Asp Gly Asp Thr Ile Tyr Asn Gly Asn Phe
50 55 60

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Ile Ala Tyr
65 70 75 80

10 Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
85 90 95

Thr Ser Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
100 105 110

15 Leu Val Thr Val Ser Ala
115

20 <210> 153
<211> 112
<212> PRT
<213> Mus musculus

25 <400> 153
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Leu Pro Val Thr Pro Gly
1 5 10 15

30 Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
35 40 45

35 Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
 65 70 75 80

5 Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
 85 90 95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
 100 105 110

10

<210> 154
 <211> 423
 <212> DNA
 15 <213> Mus musculus

<220>
 <221> CDS
 <222> (1).. (423)
 20 <223>

<400> 154
 atg gtt ctt gcc agc tct acc acc agc atc cac acc atg ctg ctc ctg 48
 Met Val Leu Ala Ser Ser Thr Thr Ser Ile His Thr Met Leu Leu Leu

25 1 5 10 15

ctc ctg atg ctg gcc cag ccg gcc atg gcg gaa gtg aag ctg gtg gag 96
 Leu Leu Met Leu Ala Gln Pro Ala Met Ala Glu Val Lys Leu Val Glu

20 25 30

30

tct ggg gga ggc tta gtg aag cct gga ggg tcc cgg aaa ctc tcc tgt 144
 Ser Gly Gly Gly Leu Val Lys Pro Gly Gly Ser Arg Lys Leu Ser Cys

35 40 45

35 gca gcc tct gga ttc act ttc agt agc tat acc atg tct tgg gtt cgc 192
 Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr Thr Met Ser Trp Val Arg

50 55 60

cag act ccg gcg aag agg ctg gag tgg gtc gca acc att agt agt ggc 240
Gln Thr Pro Ala Lys Arg Leu Glu Trp Val Ala Thr Ile Ser Ser Gly
5 65 70 75 80

agt agt acc atc tac tat gca gac aca gtg aag ggc cga ttc acc atc 288
Ser Ser Thr Ile Tyr Tyr Ala Asp Thr Val Lys Gly Arg Phe Thr Ile
85 90 95

10 tcc aga gac aat gcc aag aac acc ctg ttc ctg caa atg acc agt cta 336
Ser Arg Asp Asn Ala Lys Asn Thr Leu Phe Leu Gln Met Thr Ser Leu
100 105 110

15 agg tot gag gac aca gcc atg tat tac tgt gca agg aga tgg ttt ott 384
Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys Ala Arg Arg Trp Phe Leu
115 120 125

gac tgc tgg ggc caa ggc acc act ctc aca gtc tcc tcg 423
20 Asp Cys Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser
130 135 140

<210> 155
25 <211> 141
<212> PRT
<213> Mus musculus

<400> 155
30 Met Val Leu Ala Ser Ser Thr Thr Ser Ile His Thr Met Leu Leu Leu
1 5 10 15

Leu Leu Met Leu Ala Gln Pro Ala Met Ala Glu Val Lys Leu Val Glu
20 25 30

35 Ser Gly Gly Gly Leu Val Lys Pro Gly Gly Ser Arg Lys Leu Ser Cys

35 40 45

Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr Thr Met Ser Trp Val Arg
50 55 60

5

Gln Thr Pro Ala Lys Arg Leu Glu Trp Val Ala Thr Ile Ser Ser Gly
65 70 75 80

Ser Ser Thr Ile Tyr Tyr Ala Asp Thr Val Lys Gly Arg Phe Thr Ile
10 85 90 95

Ser Arg Asp Asn Ala Lys Asn Thr Leu Phe Leu Gln Met Thr Ser Leu
100 105 110

15 Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys Ala Arg Arg Trp Phe Leu
115 120 125

Asp Cys Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser
130 135 140

20

<210> 156
<211> 357
<212> DNA
25 <213> Mus musculus

<220>
<221> CDS
<222> (1).. (357)
30 <223>

<400> 156
gat att gtg ctc acc caa tct cca gct tct ttg gct gtg tct cta ggg 48
Asp Ile Val Leu Thr Gln Ser Pro Ala Ser Leu Ala Val Ser Leu Gly
35 1 5 10 15

	cag agt gtc acc atc tcc tgc aga gcc agt gaa agt gtt gaa tat tat	96
	Gln Ser Val Thr Ile Ser Cys Arg Ala Ser Glu Ser Val Glu Tyr Tyr	
	20 25 30	
5	ggc act agt tta atg cag tgg tac caa cag aaa cca gga cag cca ccc	144
	Gly Thr Ser Leu Met Gln Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro	
	35 40 45	
10	aaa ctc ctc atc tat ggt gca tcc aac gta gaa tct ggg gtc cct gcc	192
	Lys Leu Leu Ile Tyr Gly Ala Ser Asn Val Glu Ser Gly Val Pro Ala	
	50 55 60	
15	agg ttt agt ggc agt ggg tct ggg aca gac ttc agc ctc aac atc cat	240
	Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Ser Leu Asn Ile His	
	65 70 75 80	
20	cct gtg gag gag gat gat att gca atg tat ttc tgt cag caa agt agg	288
	Pro Val Glu Glu Asp Asp Ile Ala Met Tyr Phe Cys Gln Gln Ser Arg	
	85 90 95	
25	aag gtt ccg tgg acg ttc ggt gga ggc acc aag ctg gaa ata aag gac	336
	Lys Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Asp	
	100 105 110	
30	tac aag gat gac gac gat aag	357
	Tyr Lys Asp Asp Asp Asp Lys	
	115	
35	<210> 157	
	<211> 119	
	<212> PRT	
	<213> Mus musculus	
35	<400> 157	
	Asp Ile Val Leu Thr Gln Ser Pro Ala Ser Leu Ala Val Ser Leu Gly	

1 5 10 15
Gln Ser Val Thr Ile Ser Cys Arg Ala Ser Glu Ser Val Glu Tyr Tyr
 20 25 30
5
Gly Thr Ser Leu Met Gln Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro
 35 40 45
Lys Leu Leu Ile Tyr Gly Ala Ser Asn Val Glu Ser Gly Val Pro Ala
10 50 55 60
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Ser Leu Asn Ile His
65 70 75 80
15 Pro Val Glu Glu Asp Asp Ile Ala Met Tyr Phe Cys Gln Gln Ser Arg
 85 90 95
Lys Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Asp
 100 105 110
20
Tyr Lys Asp Asp Asp Asp Lys
 115

25 <210> 158
<211> 432
<212> DNA
<213> Mus musculus

30 <220>
<221> CDS
<222> (1)..(432)
<223>

35 <400> 158
atg gtt ctt gcc agc tct acc acc agc atc cac acc atg ctg ctc ctg

	Met	Val	Leu	Ala	Ser	Ser	Thr	Thr	Ser	Ile	His	Thr	Met	Leu	Leu	Leu	
	1				5					10					15		
	ctc ctg atg ctg gcc cag ccg gcc atg gcg cag gtt cag ctc cag caa																96
5	Leu	Leu	Met	Leu	Ala	Gln	Pro	Ala	Met	Ala	Gln	Val	Gln	Leu	Gln	Gln	
				20				25					30				
	tct gga cct gag ctg gtg aag cct ggg gcc tca gtg aag att tcc tgc																144
	Ser	Gly	Pro	Glu	Leu	Val	Lys	Pro	Gly	Ala	Ser	Val	Lys	Ile	Ser	Cys	
10			35				40					45					
	aag gct tct ggc tat gca ttc agt agc tcc tgg atg aac tgg atg aag																192
	Lys	Ala	Ser	Gly	Tyr	Ala	Phe	Ser	Ser	Ser	Trp	Met	Asn	Trp	Met	Lys	
		50				55				60							
15	cag agg cct gga aag ggt ctt gag tgg att ggg cgg att tat cct gga																240
	Gln	Arg	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Ile	Gly	Arg	Ile	Tyr	Pro	Gly	
	65				70				75				80				
	gat gga gat act aac tac aat ggg aag ttc aag ggc aag gcc aca ctg																288
20	Asp	Gly	Asp	Thr	Asn	Tyr	Asn	Gly	Lys	Phe	Lys	Gly	Lys	Ala	Thr	Leu	
				85				90				95					
	act gca gac aaa tcc tcc agc aca gcc tac atg caa ctc agc agc ctg																336
25	Thr	Ala	Asp	Lys	Ser	Ser	Ser	Thr	Ala	Tyr	Met	Gln	Leu	Ser	Ser	Leu	
				100				105				110					
	aca tct gag gac tct gcg gtc tac ttc tgt gca aga gcg agg aaa act																384
	Thr	Ser	Glu	Asp	Ser	Ala	Val	Tyr	Phe	Cys	Ala	Arg	Ala	Arg	Lys	Thr	
30			115				120					125					
	tcc tgg ttt gct tac tgg ggc caa ggg act ctg gtc act gtc tct gcg																432
	Ser	Trp	Phe	Ala	Tyr	Trp	Gly	Gln	Gly	Thr	Leu	Val	Thr	Val	Ser	Ala	
		130				135				140							

<210> 159

<211> 144

<212> PRT

<213> Mus musculus

5

<400> 159

Met Val Leu Ala Ser Ser Thr Thr Ser Ile His Thr Met Leu Leu Leu
1 5 10 15

10 Leu Leu Met Leu Ala Gln Pro Ala Met Ala Gln Val Gln Leu Gln Gln
20 25 30

Ser Gly Pro Glu Leu Val Lys Pro Gly Ala Ser Val Lys Ile Ser Cys
35 40 45

15

Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser Trp Met Asn Trp Met Lys
50 55 60

20 Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile Gly Arg Ile Tyr Pro Gly
65 70 75 80

Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe Lys Gly Lys Ala Thr Leu
85 90 95

25 Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu
100 105 110

Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Ala Arg Ala Arg Lys Thr
115 120 125

30

Ser Trp Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala
130 135 140

35 <210> 160

<211> 345

<212> DNA

<213> Mus musculus

<220>

5 <221> CDS

<222> (1)..(345)

<223>

<400> 160

10 gac att gtg ttg aca cag tct caa aaa ttc atg tcc aca tca gta gga 48
 Asp Ile Val Leu Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
 1 5 10 15

gac agg gtc agc atc agc tgc aag gcc agt cag aat gtg ggt aat att 96
 15 Asp Arg Val Ser Ile Ser Cys Lys Ala Ser Gln Asn Val Gly Asn Ile
 20 25 30

ata gcc tgg tat caa cag aaa cca ggg caa tct cct aaa gca ctg att 144
 Ile Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile
 20 35 40 45

tac ttg gca tcc tac cgg tac agt gga gtc cct gat cgc ttc aca ggc 192
 Tyr Leu Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly
 50 55 60

25 agt gga tct ggg aca gat ttc act ctc acc att agt aat gtg cag tct 240
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln Ser
 65 70 75 80

30 gaa gac ttg gca gag tat ttc tgt cag caa tat agc agc tct ccg ctc 288
 Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Ser Ser Ser Pro Leu
 85 90 95

acg ttc ggt gct ggg acc aag ctg gaa ata aag gac tac aag gat gac 336
 35 Thr Phe Gly Ala Gly Thr Lys Leu Glu Ile Lys Asp Tyr Lys Asp Asp
 100 105 110

gac gat aag 345
Asp Asp Lys
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10 <213> Mus musculus
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Ile Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile
20 35 40 45
Tyr Leu Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly
50 55 60
25 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln Ser
65 70 75 80
Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Ser Ser Ser Pro Leu
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30 Thr Phe Gly Ala Gly Thr Lys Leu Glu Ile Lys Asp Tyr Lys Asp Asp
100 105 110
Asp Asp Lys
35 115

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<211> 116

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5 <213> Mus musculus

<400> 162

Asp Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Gln
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Tyr Ala Trp Ser Trp Ile Arg Gln Leu Pro Gly Asn Lys Leu Glu Trp
35 40 45

Met Gly Tyr Ile Thr Tyr Ser Gly Tyr Ser Ile Tyr Asn Pro Ser Leu
50 55 60

20

Lys Ser Arg Ile Ser Ile Ser Arg Asp Thr Ser Lys Asn Gln Leu Phe
65 70 75 80

Leu Gln Leu Asn Ser Val Thr Thr Glu Asp Thr Ala Thr Tyr Tyr Cys
85 90 95

25

Val Gly Gly Tyr Asp Asn Met Asp Tyr Trp Gly Gln Gly Thr Ser Val
100 105 110

30

Thr Val Ser Ser
115

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<211> 108

35 <212> PRT

<213> Mus musculus

<400> 163

Gln Ile Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly

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Glu Lys Val Thr Leu Thr Cys Ser Ala Ser Ser Ser Val Ser Ser Ser

20 25 30

His Leu Tyr Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Lys Leu Trp

10 35 40 45

Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro Ala Arg Phe Ser

50 55 60

15 Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Asn Met Glu

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85 90 95

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100 105

25 <210> 164

<211> 1924

<212> DNA

<213> *Macaca fascicularis*

30 <220>

<221> CDS

<222> (11).. (1918)

<223>

35 <400> 164

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	ctc	ctg	gcc	cct	caa	aac	ctg	gcc	caa	gtc	agc	agc	caa	gat	gtc	tcc	97
5	Leu	Leu	Ala	Pro	Gln	Asn	Leu	Ala	Gln	Val	Ser	Ser	Gln	Asp	Val	Ser	
	15				20					25							
	ttg	ctg	gcc	tgc	gac	tca	gag	ccc	ctg	aag	tgt	ttc	tcc	cga	aca	ttt	145
	Leu	Leu	Ala	Ser	Asp	Ser	Glu	Pro	Leu	Lys	Cys	Phe	Ser	Arg	Thr	Phe	
10	30				35					40						45	
	gag	gac	ctc	act	tgc	ttc	tgg	gat	gag	gaa	gag	gca	gca	ccc	agt	ggg	193
	Glu	Asp	Leu	Thr	Cys	Phe	Trp	Asp	Glu	Glu	Glu	Ala	Ala	Pro	Ser	Gly	
				50					55					60			
15	aca	tac	cag	ctg	ctg	tat	gcc	tac	ccg	ggg	gag	aag	ccc	cgt	gcc	tgc	241
	Thr	Tyr	Gln	Leu	Leu	Tyr	Ala	Tyr	Pro	Gly	Glu	Lys	Pro	Arg	Ala	Cys	
				65					70					75			
	ccc	ctg	agt	tct	cag	agc	gtg	ccc	cgc	ttt	gga	acc	cga	tac	gtg	tgc	289
20	Pro	Leu	Ser	Ser	Gln	Ser	Val	Pro	Arg	Phe	Gly	Thr	Arg	Tyr	Val	Cys	
		80					85						90				
	cag	ttt	cca	gcc	cag	gaa	gaa	gtg	cgt	ctc	ttc	tct	ccg	ctg	cac	ctc	337
25	Gln	Phe	Pro	Ala	Gln	Glu	Glu	Val	Arg	Leu	Phe	Ser	Pro	Leu	His	Leu	
		95				100							105				
	tgg	gtg	aag	aat	gtg	ttc	cta	aac	cag	act	cag	att	cag	cga	gtc	ctc	385
	Trp	Val	Lys	Asn	Val	Phe	Leu	Asn	Gln	Thr	Gln	Ile	Gln	Arg	Val	Leu	
30	110				115					120					125		
	ttt	gtg	gac	agt	gta	ggc	ctg	ccg	gct	ccc	ccc	agt	atc	atc	aag	gcc	433
	Phe	Val	Asp	Ser	Val	Gly	Leu	Pro	Ala	Pro	Pro	Ser	Ile	Ile	Lys	Ala	
					130					135					140		
35	atg	ggt	ggg	agc	cag	cca	ggg	gaa	ctt	cag	atc	agc	tgg	gag	gcc	cca	481

	Met Gly Gly Ser Gln Pro Gly Glu Leu Gln Ile Ser Trp Glu Ala Pro	
	145 150 155	
	gct cca gaa atc agt gat ttc ctg agg tac gaa ctc cgc tat ggc ccc	529
5	Ala Pro Glu Ile Ser Asp Phe Leu Arg Tyr Glu Leu Arg Tyr Gly Pro	
	160 165 170	
	aaa gat ctc aag aac tcc act ggt ccc acg gtc ata cag ttg atc gcc	577
10	Lys Asp Leu Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala	
	175 180 185	
	aca gaa acc tgc tgc cct gct ctg cag agg cca cac tca gcc tct gct	625
15	Thr Glu Thr Cys Cys Pro Ala Leu Gln Arg Pro His Ser Ala Ser Ala	
	190 195 200 205	
	ctg gac cag tct cca tgt gct cag ccc aca atg ccc tgg caa gat gga	673
	Leu Asp Gln Ser Pro Cys Ala Gln Pro Thr Met Pro Trp Gln Asp Gly	
	210 215 220	
20	cca aag cag acc tcc cca act aga gaa gct tca gct ctg aca gca gtg	721
	Pro Lys Gln Thr Ser Pro Thr Arg Glu Ala Ser Ala Leu Thr Ala Val	
	225 230 235	
	ggt gga agc tgc ctc atc tca gga ctc cag cct ggc aac tcc tac tgg	769
25	Gly Gly Ser Cys Leu Ile Ser Gly Leu Gln Pro Gly Asn Ser Tyr Trp	
	240 245 250	
	ctg cag ctg cgc agc gaa cct gat ggg atc tcc ctc ggt ggc tcc tgg	817
30	Leu Gln Leu Arg Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp	
	255 260 265	
	gga tcc tgg tcc ctc cct gtg act gtg gac ctg cct gga gat gca gtg	865
35	Gly Ser Trp Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val	
	270 275 280 285	
	gca att gga ctg caa tgc ttt acc ttg gac ctg aag aat gtt acc tgt	913

	Ala Ile Gly Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys	
	290 295 300	
	caa tgg cag caa gag gac cat gct agt tcc caa ggt ttc ttc tac cac	961
5	Gln Trp Gln Gln Glu Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His	
	305 310 315	
	agc agg gca cgg tgc tgc ccc aga gac agg tac ccc atc tgg gag gac	1009
10	Ser Arg Ala Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asp	
	320 325 330	
	tgt gaa gag gaa gag aaa aca aat cca gga tta cag acc cca cag ttc	1057
15	Cys Glu Glu Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe	
	335 340 345	
	tct cgc tgc cac ttc aag tca cga aat gac agc gtt att cac atc ctt	1105
	Ser Arg Cys His Phe Lys Ser Arg Asn Asp Ser Val Ile His Ile Leu	
	350 355 360 365	
20	gtg gag gtg acc aca gcc ctg ggt gct gtt cac agt tac ctg ggc tcc	1153
	Val Glu Val Thr Thr Ala Leu Gly Ala Val His Ser Tyr Leu Gly Ser	
	370 375 380	
	cct ttc tgg atc cac cag gct gtg cgc ctc ccc acc cca aac ttg cac	1201
25	Pro Phe Trp Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His	
	385 390 395	
	tgg agg gag atc tcc agc ggg cat ctg gaa ttg gag tgg cag cac cca	1249
30	Trp Arg Glu Ile Ser Ser Gly His Leu Glu Leu Glu Trp Gln His Pro	
	400 405 410	
	tca tcc tgg gca gcc caa gag acc tgc tat caa ctc cga tac aca gga	1297
35	Ser Ser Trp Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly	
	415 420 425	
	gaa ggc cat cag gac tgg aag gtg ctg gag ccg cct ctc ggg gcc cga	1345

	Glu Gly His Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg	
	430 435 440 445	
	gga ggg acc ctg gag ctg cgc ccg cga tct cgc tac cgt tta cag ctg	1393
5	Gly Gly Thr Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu	
	450 455 460	
	cgc gcc agg ctc aat ggc ccc acc tac caa ggt ccc tgg agc tcg tgg	1441
10	Arg Ala Arg Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp	
	465 470 475	
	tcg gac cca gct agg gtg gag acc gcc acc gag acc gcc tgg att tcc	1489
15	Ser Asp Pro Ala Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser	
	480 485 490	
	ttg gtg acc gct ctg ctg cta gtg ctg ggc ctc agc gcc gtc ctg ggc	1537
	Leu Val Thr Ala Leu Leu Leu Val Leu Gly Leu Ser Ala Val Leu Gly	
	495 500 505	
20	ctg ctg ctg ctg agg tgg cag ttt cct gca cac tac agg aga ctg agg	1585
	Leu Leu Leu Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg	
	510 515 520 525	
	cat gcc ctg tgg ccc tca ctt cca gat ctg cac cga gtc cta ggc cag	1633
25	His Ala Leu Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln	
	530 535 540	
	tac ctt agg gac act gca gcc ctg agt ccg ccc aag gcc aca gtc tca	1681
30	Tyr Leu Arg Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser	
	545 550 555	
	gat acc tgt gaa gaa gtg gaa ccc agc ctc ctt gaa atc ctc ccc aag	1729
35	Asp Thr Cys Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys	
	560 565 570	
	tcc tca gag agg act cct ttg ccc ctg tgt tcc tcc cag tcc cag atg	1777

Ser Ser Glu Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ser Gln Met
575 580 585

gac tac cga aga ttg cag cct tct tgc ctg ggg acc atg ccc ctg tct 1825
5 Asp Tyr Arg Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser
590 595 600 605

gtg tgc cca ccc atg gct gag tca ggg tcc tgc tgt acc acc cac att 1873
10 Val Cys Pro Pro Met Ala Glu Ser Gly Ser Cys Cys Thr Thr His Ile
610 615 620

gcc aac cat tcc tac cta cca cta agc tat tgg cag cag cct tga 1918
Ala Asn His Ser Tyr Leu Pro Leu Ser Tyr Trp Gln Gln Pro
625 630 635

15 gtcgac 1924

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20 <211> 635
<212> PRT
<213> Macaca fascicularis

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20 25 30

30 Ser Asp Ser Glu Pro Leu Lys Cys Phe Ser Arg Thr Phe Glu Asp Leu
35 40 45

Thr Cys Phe Trp Asp Glu Glu Glu Ala Ala Pro Ser Gly Thr Tyr Gln
35 50 55 60

	Leu	Leu	Tyr	Ala	Tyr	Pro	Gly	Glu	Lys	Pro	Arg	Ala	Cys	Pro	Leu	Ser
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5	Ser	Gln	Ser	Val	Pro	Arg	Phe	Gly	Thr	Arg	Tyr	Val	Cys	Gln	Phe	Pro
					85					90					95	
	Ala	Gln	Glu	Glu	Val	Arg	Leu	Phe	Ser	Pro	Leu	His	Leu	Trp	Val	Lys
					100					105					110	
10	Asn	Val	Phe	Leu	Asn	Gln	Thr	Gln	Ile	Gln	Arg	Val	Leu	Phe	Val	Asp
					115					120					125	
	Ser	Val	Gly	Leu	Pro	Ala	Pro	Pro	Ser	Ile	Ile	Lys	Ala	Met	Gly	Gly
					130					135					140	
15	Ser	Gln	Pro	Gly	Glu	Leu	Gln	Ile	Ser	Trp	Glu	Ala	Pro	Ala	Pro	Glu
					145					150					155	160
	Ile	Ser	Asp	Phe	Leu	Arg	Tyr	Glu	Leu	Arg	Tyr	Gly	Pro	Lys	Asp	Leu
20					165					170					175	
	Lys	Asn	Ser	Thr	Gly	Pro	Thr	Val	Ile	Gln	Leu	Ile	Ala	Thr	Glu	Thr
					180					185					190	
25	Cys	Cys	Pro	Ala	Leu	Gln	Arg	Pro	His	Ser	Ala	Ser	Ala	Leu	Asp	Gln
					195					200					205	
	Ser	Pro	Cys	Ala	Gln	Pro	Thr	Met	Pro	Trp	Gln	Asp	Gly	Pro	Lys	Gln
					210					215					220	
30	Thr	Ser	Pro	Thr	Arg	Glu	Ala	Ser	Ala	Leu	Thr	Ala	Val	Gly	Gly	Ser
					225					230					235	240
	Cys	Leu	Ile	Ser	Gly	Leu	Gln	Pro	Gly	Asn	Ser	Tyr	Trp	Leu	Gln	Leu
35					245					250					255	

Arg Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp Gly Ser Trp
260 265 270

5 Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val Ala Ile Gly
275 280 285

Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys Gln Trp Gln
290 295 300

10 Gln Glu Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His Ser Arg Ala
305 310 315 320

Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asp Cys Glu Glu
325 330 335

15 Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe Ser Arg Cys
340 345 350

His Phe Lys Ser Arg Asn Asp Ser Val Ile His Ile Leu Val Glu Val
355 360 365

20 Thr Thr Ala Leu Gly Ala Val His Ser Tyr Leu Gly Ser Pro Phe Trp
370 375 380

25 Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His Trp Arg Glu
385 390 395 400

Ile Ser Ser Gly His Leu Glu Leu Glu Trp Gln His Pro Ser Ser Trp
405 410 415

30 Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly Glu Gly His
420 425 430

Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg Gly Gly Thr
435 440 445

35

Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu Arg Ala Arg
450 455 460

Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp Ser Asp Pro
5 465 470 475 480

Ala Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser Leu Val Thr
485 490 495

Ala Leu Leu Leu Val Leu Gly Leu Ser Ala Val Leu Gly Leu Leu Leu
10 500 505 510

Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg His Ala Leu
515 520 525

15 Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln Tyr Leu Arg
530 535 540

Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser Asp Thr Cys
20 545 550 555 560

Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys Ser Ser Glu
565 570 575

25 Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ser Gln Met Asp Tyr Arg
580 585 590

Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser Val Cys Pro
595 600 605

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610 615 620

Ser Tyr Leu Pro Leu Ser Tyr Trp Gln Gln Pro
35 625 630 635

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<211> 24
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5 <213> Artificial

<220>
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10 <400> 166
caggggccag tggatagact gatg 24

<210> 167
15 <211> 23
<212> DNA
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<220>
20 <223> an artificially synthesized sequence

<400> 167
gctcactgga tggtaggaag atg 23

25
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<211> 30
<212> DNA
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<400> 168
35 tagaattcca ccatggaatg gcctttgatc 30

<210> 169

<211> 56

<212> DNA

5 <213> Artificial

<220>

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10 <400> 169

agcctgagtc atcacaatat ccgatccgcc tccacctgca gagacagtga ccagag 56

<210> 170

15 <211> 56

<212> DNA

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<220>

20 <223> an artificially synthesized primer sequence

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actctgggtca ctgtctctgc aggtggaggc ggatcggata ttgtgatgac tcaggc 56

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<210> 171

<211> 60

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Asp Tyr Lys Asp Asp Asp Asp Lys

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15 <210> 173

<211> 85

<212> DNA

<213> Artificial

20 <220>

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tagaattcca ccatggaatg gcctttgatc tttctcttcc tcctgtcagg aactgcaggt 60

25

gtccactccc aggttcagct gcagc 85

<210> 174

30 <211> 82

<212> DNA

<213> Artificial

<220>

35 <223> an artificially synthesized primer sequence

<400> 174

tggtcactgt ctctgcaggt ggtggtggtt cgggtggtgg tggttcgggt ggtggcggat 60

cggatattgt gatgactcag gc 82

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<210> 175

<211> 82

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10 <213> Artificial

<220>

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15 <400> 175

tgagtcatca caatatccga tccgccacca cccgaaccac caccaccoga accaccacca 60

cctgcagaga cagtgaccag ag 82

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<210> 176

<211> 25

<212> DNA

<213> Artificial

25

<220>

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30 caggttcagc tgcagcagtc tggac 25

<210> 177

<211> 81

35 <212> DNA

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<220>

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5 <400> 177

gctgcagctg aacctgcgat ccaccgcctc ccgaaccacc accaccgat ccaccacctc 60

cttttatttc cagcttggtc c 81

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<211> 38

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<400> 178

20 gccagccgg ccatggcgga kgtrmagctt caggagtc 38

<210> 179

<211> 38

25 <212> DNA

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<400> 179

gccagccgg ccatggcgga ggtbcagctb cagcagtc 38

35 <210> 180

<211> 38

<212> DNA

<213> Artificial

<220>

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<400> 180

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<210> 181

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25 <212> DNA

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35 <210> 183

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<210> 184

<211> 38

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20 gcccagccgg ccatggcgca ggtccacgtg aagcagtc

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<211> 38

25 <212> DNA

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<400> 185

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35 <210> 186

<211> 38

<212> DNA

<213> Artificial

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5 <223> an artificially synthesized primer sequence

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gccagccgg ccatggcgga vgtgawgytg gtggagtc

38

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<211> 38

<212> DNA

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20 gccagccgg ccatggcgga ggtgcagskg gtggagtc

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<210> 188

<211> 38

25 <212> DNA

<213> Artificial

<220>

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<400> 188

gccagccgg ccatggcgga kgtgcamctg gtggagtc

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35 <210> 189

<211> 38

<212> DNA

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gccagccgg ccatggcgga ggtgaagctg atggartc

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<211> 38

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25 <212> DNA

<213> Artificial

<220>

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35 <210> 192

<211> 38

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<220>

5 <223> an artificially synthesized primer sequence

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38

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<211> 40

<212> DNA

<213> Artificial

15

<220>

<223> an artificially synthesized primer sequence

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<210> 194

<211> 38

25 <212> DNA

<213> Artificial

<220>

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<400> 194

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38

35 <210> 195

<211> 38

<212> DNA

<213> Artificial

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5 <223> an artificially synthesized primer sequence

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38

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<210> 196

<211> 38

<212> DNA

<213> Artificial

15

<220>

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<400> 196

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38

<210> 197

<211> 36

25 <212> DNA

<213> Artificial

<220>

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<400> 197

ggagccgccg ccgcccagg aaacggtgac cgtggt

36

35 <210> 198

<211> 36

<212> DNA

<213> Artificial

<220>

5 <223> an artificially synthesized primer sequence

<400> 198

ggagccgccg ccgcccgagg agactgtgag agtggt

36

10

<210> 199

<211> 36

<212> DNA

<213> Artificial

15

<220>

<223> an artificially synthesized primer sequence

<400> 199

20 ggagccgccg ccgcccgcag agacagtgac cagagt

36

<210> 200

<211> 36

25 <212> DNA

<213> Artificial

<220>

<223> an artificially synthesized primer sequence

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<400> 200

ggagccgccg ccgcccgagg agacggtgac tgaggt

36

35 <210> 201

<211> 35

<212> DNA

<213> Artificial

<220>

5 <223> an artificially synthesized primer sequence

<400> 201

ggcggcggcg gctccgayat ccagctgact cagcc

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<210> 202

<211> 35

<212> DNA

<213> Artificial

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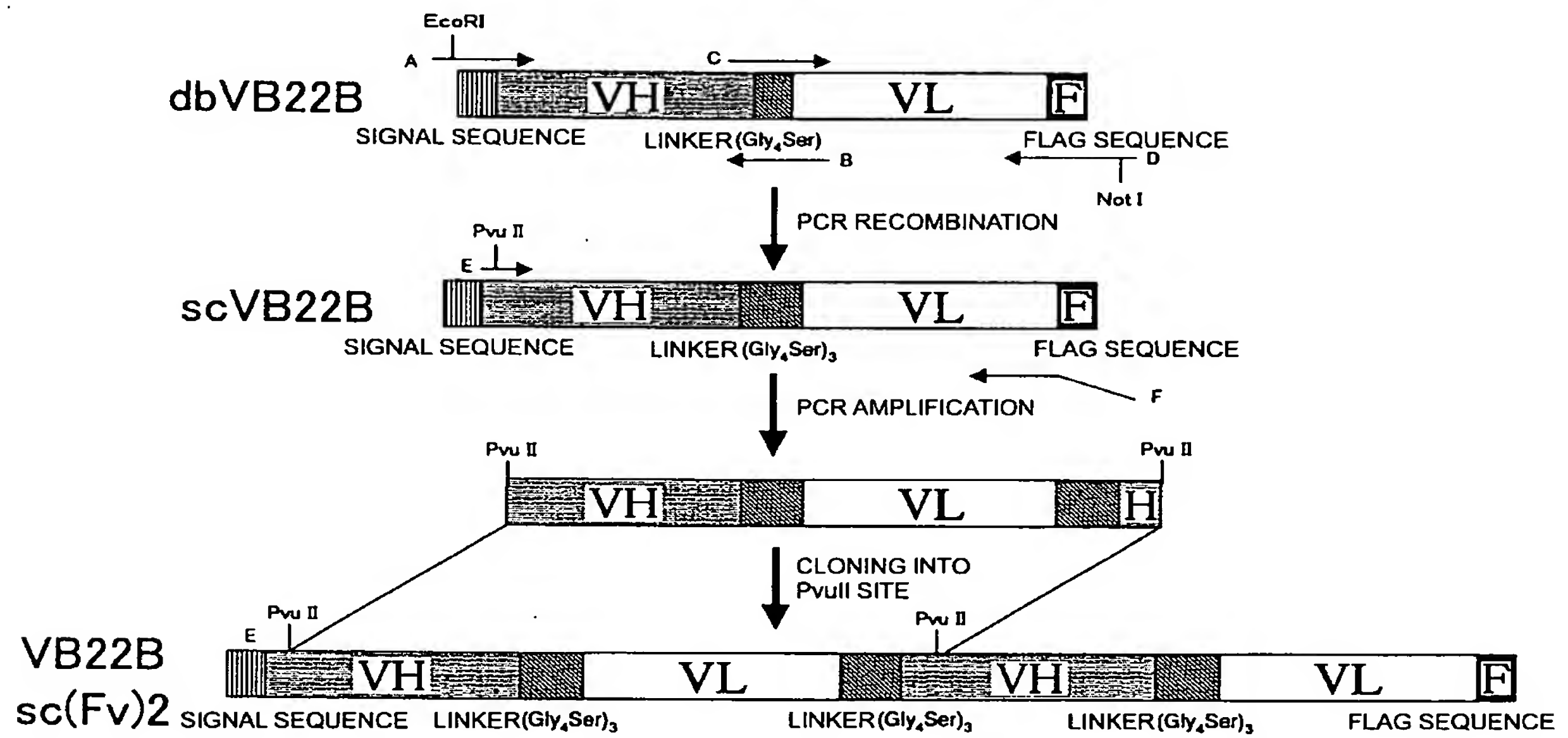
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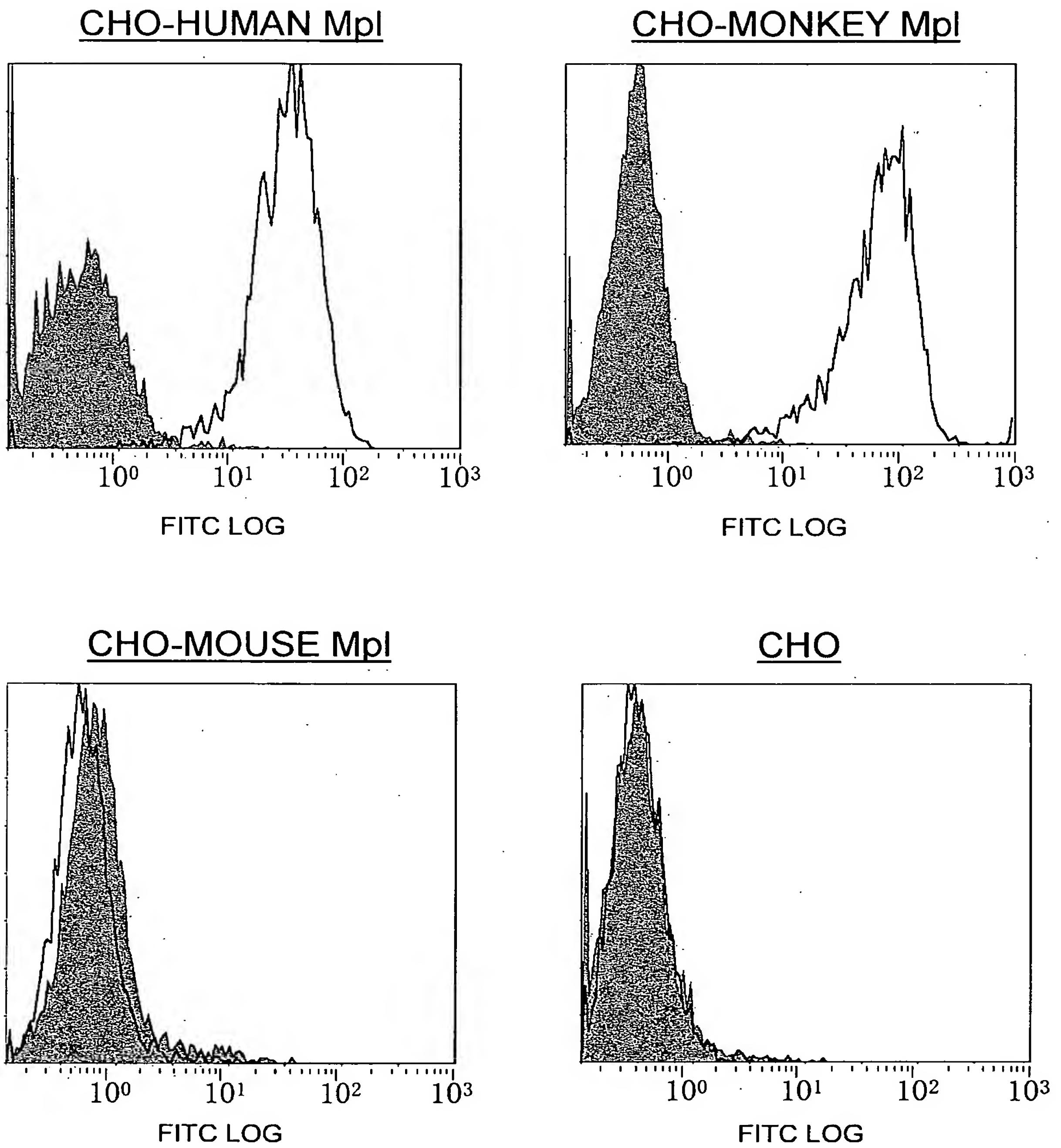
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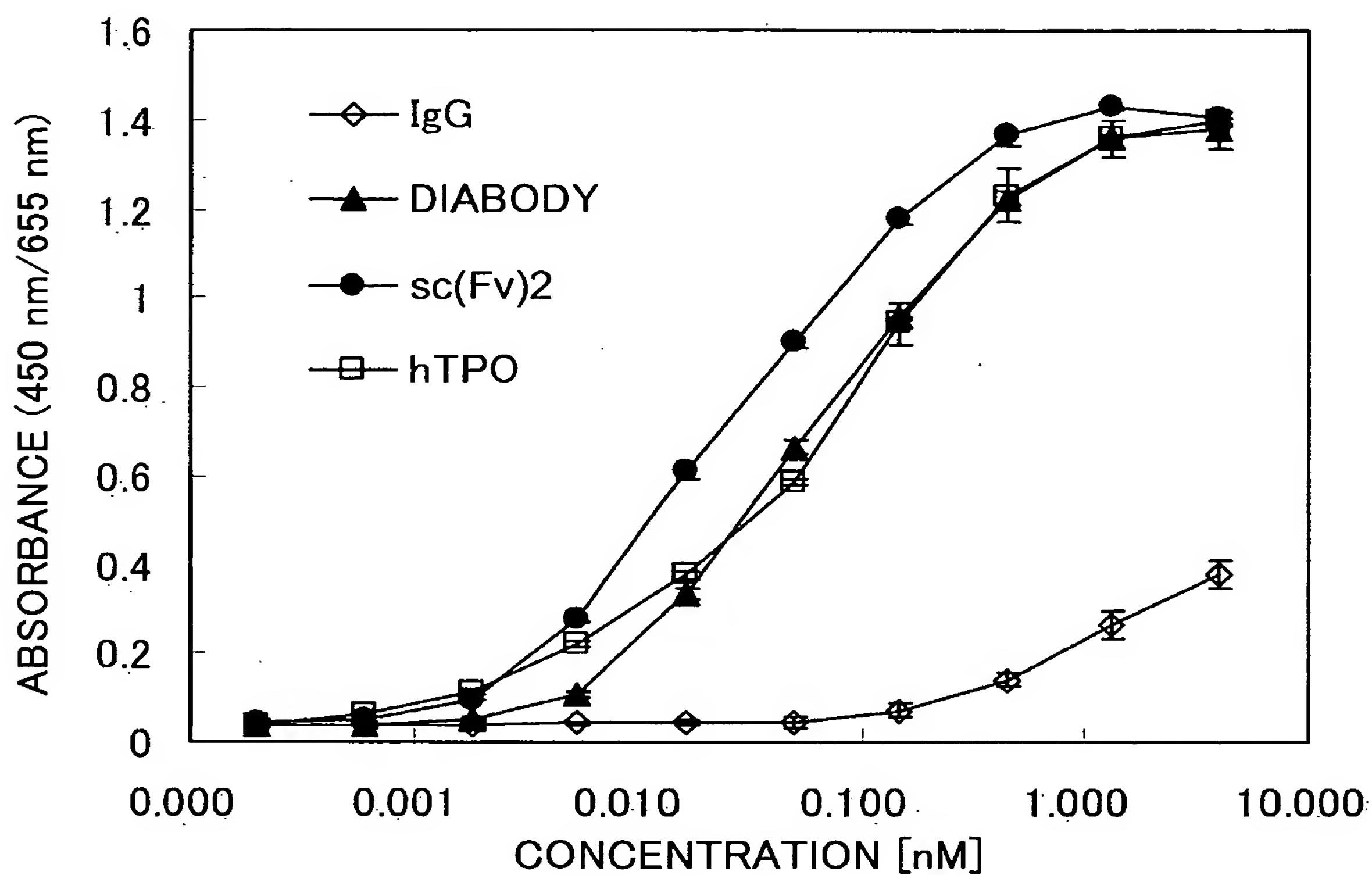
[Fig. 1]



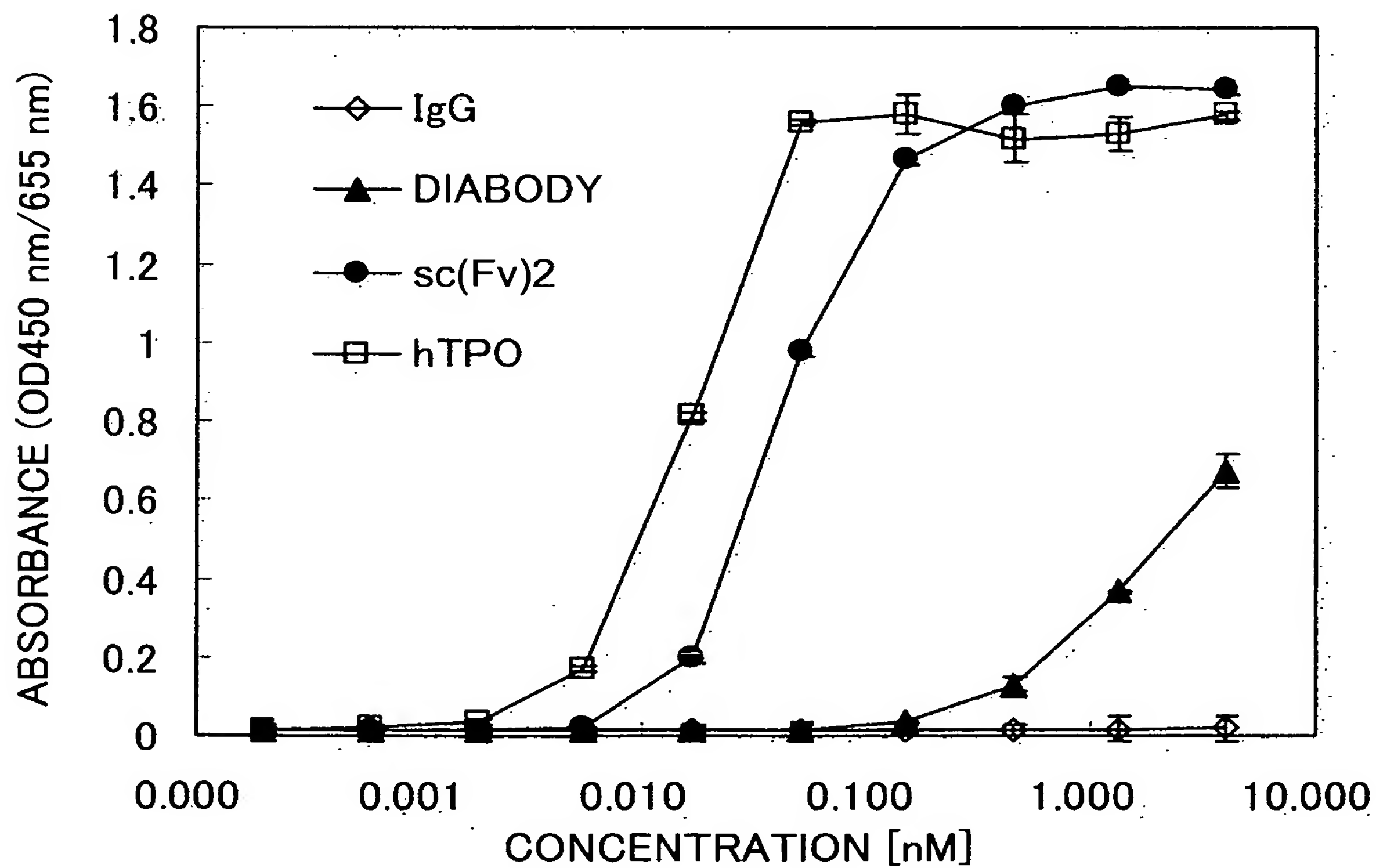
[Fig. 2]



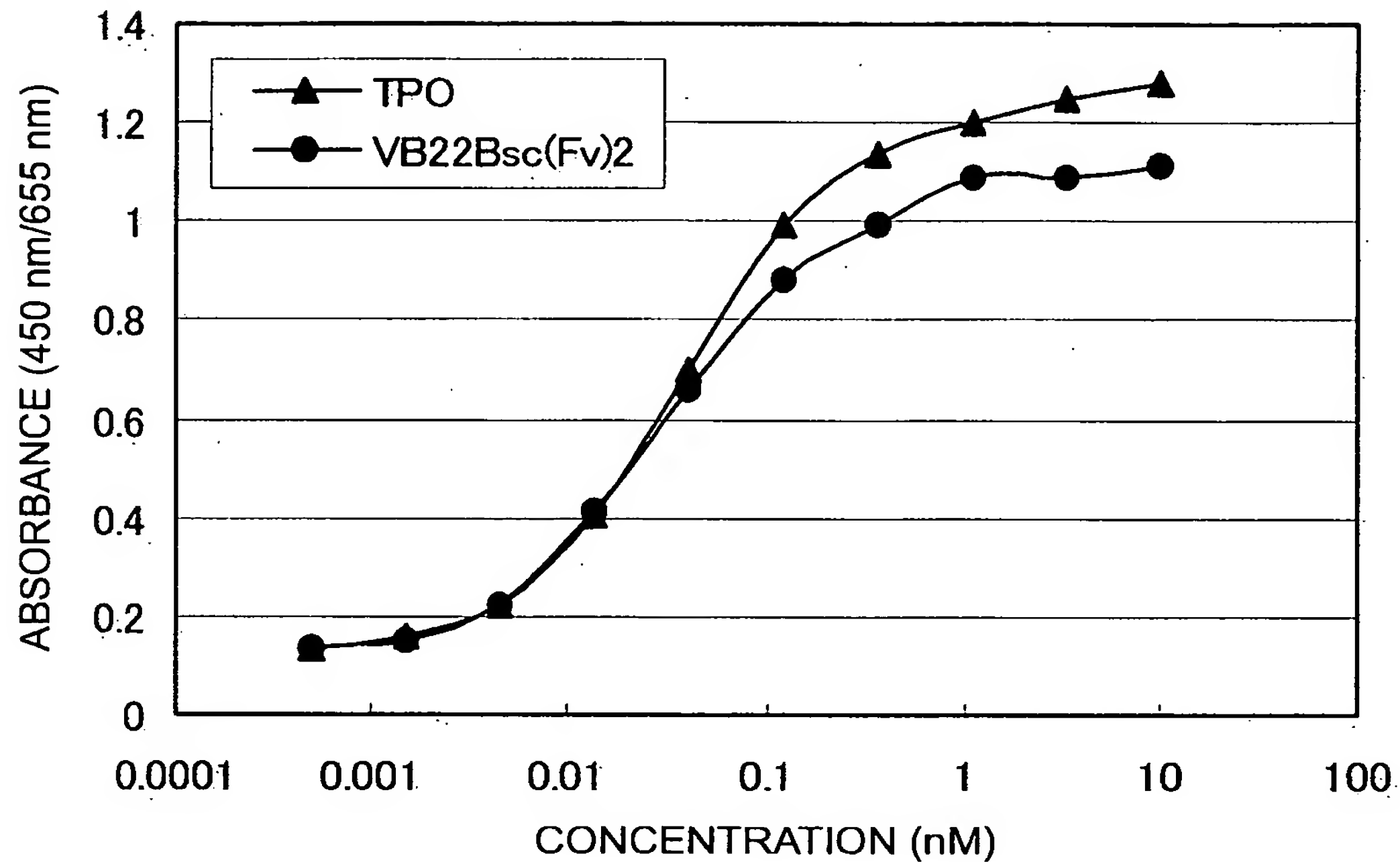
[Fig. 3]



[Fig. 4]



[Fig. 5]



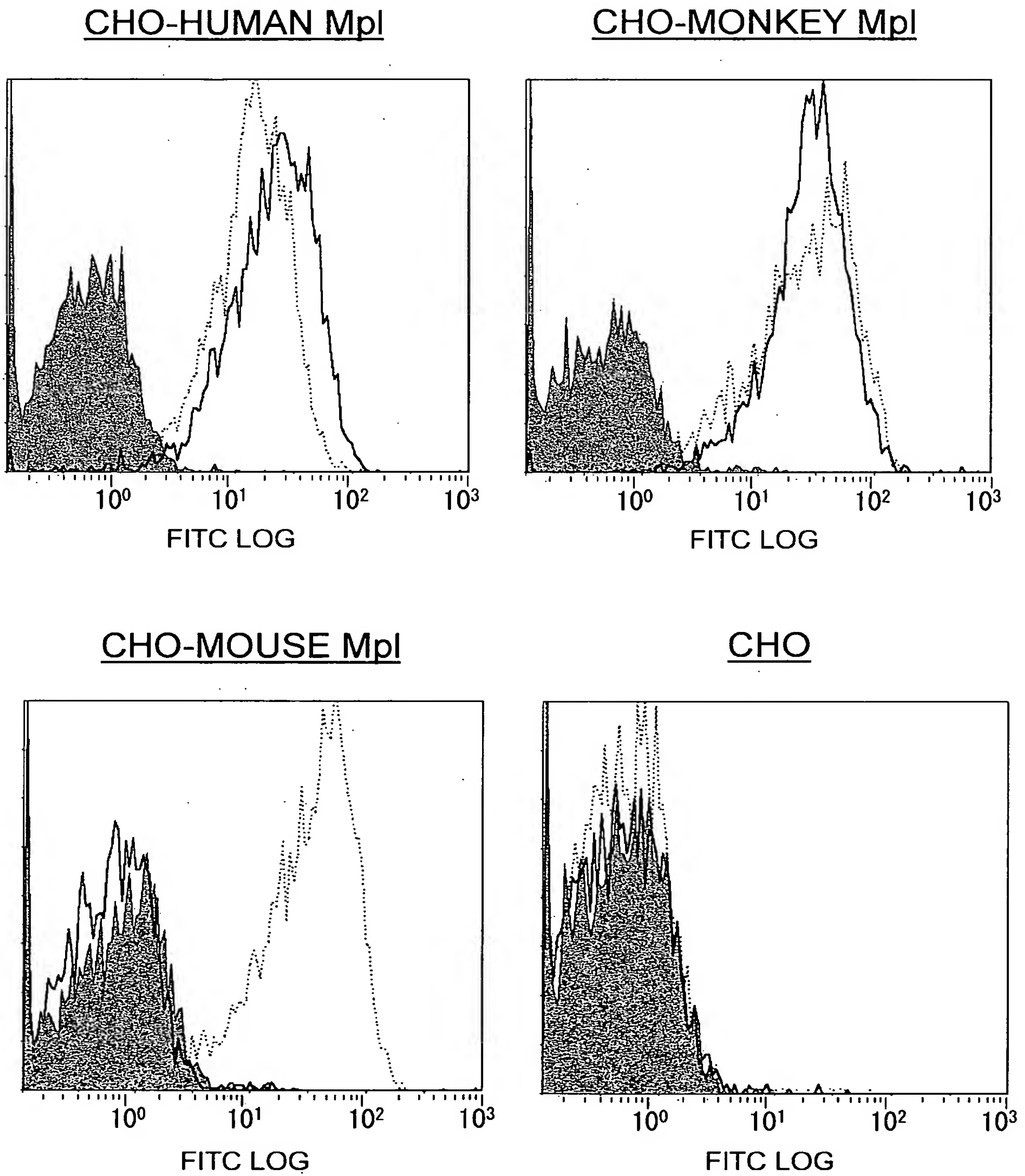
[Fig. 6]

		CDR1	CDR2
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VA130	QVQLQQSGPELVKPGASVKISCKASGYAFS	SSWMN	WVKQRPKGKLEWIG
VA259	QVQLQQSGPELVKPGASVKISCKASGYAFS	SSWMN	WVKQRPKGKLEWIG
VB17B	QVQLQQSGPELVKPGASVKISCKASGYTFS	SSWMN	WVKQRPKGKLEWIG
VB12B	QVQLQQSGPELVKPGASVKISCKASGYAFS	RSWMN	WVKQRPKGKLEWIG
VB140	QVQLQQSGPELVKPGASVKISCRAFGYAFS	NSWMN	WVKQRPKGKLEWIG
VB33	QVQLQQPGAELVKPGASVKLSCKASGYTFT	NYWVN	WVKQRPGRGLEWIG
VB45B	QVQLQQSGPELVKPGASVKISCKASGYAFS	SSWMN	WVKQRPKGKLEWIG
VB8B	QVQLQQSGPELVKPGASVKISCKASGYAFS	TSWMN	WVKQRPKGKLEWIG
VB115	QVQLQQSGPELVKPGASVKISCKASGYAFS	SSWMN	WVKQRPKGKPEWIG
VB14B	QVQLQQSGPELVKPGASVKISCKASGYAFS	RSWMN	WVKQRPKGKLEWIG
VB22B	QVQLQQSGPELVKPGASVKISCKASGYAFT	NSWMN	WVKQRPKGKLEWIG
VB16	QVQLQQPGTELVRPGASVKLSCKASGYTFT	DYWVN	WVKQRPGRGLEWIG
VB157	QVQLQQPGAELVKPGASVKLSCKASGYTFT	DYWMN	WVKQRPGRGLEWIG
VB4B	QVQLQQSGPELVKPGASVKISCKASGYAFT	NSWMN	WVRQRPKGKLEWIG
VB51	QVQLQQSGPELVKPGASVKISCKASGYAFS	NSWMN	WVNQRPKGKLEWIG
		CDR3	
VA7	KATLTADKSSSTAYMQLSSLTSEDSAVYFCAR	GWILADGGYSFAY	WGQGTLLVTVSA
VA130	KATLTADKSSSTAYIQLSSLTSEDSAVYFCAR	GYAD----YSFAY	WGQGTLLVTVSA
VA259	KATLTADKSSNTAYMQLSSLTSEDSAVYFCAR	GFGD----YSFAY	WGQGTLLVTVSA
VB17B	KATLTADKSSSTAYMQLSSLTSEDSAVYFCAS	GYAD----YSFAY	WGQGTLLVTVSA
VB12B	KATLTADKSSSTAYMQLSSLTSEDSAVYFCAS	GYDD----YSFAY	WGQGTLLVTVSA
VB140	KATLTADKSSSTAYMQLSSLTSEDSAVYFCAR	GYGD----YSFAY	WGQGTLLVTVSA
VB33	KATLTVNKSSSTAYIQLHSLTSEDSAVYYCTS	GGW-----FAY	WGQGTLLVTVSA
VB45B	KATLTADKSSSTAYMQLSSLTSEDSAVYFCAR	GYGD----YSFAY	WGQGTLLVTVSA
VB8B	KATLTADKSSSSAYMQLSSLTSEDSAVYFCAR	GYGD----YSFAY	WGQGTLLVTVSA
VB115	KATLTADKSSSTVYMQLSSLTSEDSAVYFCAR	GYGD----YSFAY	WGQGTLLVTVSA
VB14B	KATLTADKSSSTAYMQFSSLTSEDSAVYFCAR	GDGD----YSFAY	WGQGTLLVTVSA
VB22B	KATLTADKSSSTAYMDISLTSEDSAVYFCAR	GYDD----YSFAY	WGQGTLLVTVSA
VB16	KATLTVDKSSSTAYIQLSSLTSEDSAVYYCAS	GGW-----FAS	WGQGTLLVTVSA
VB157	KATLTVDKSSNTAYIQFSSLTSEDSAVYYCSS	GGW-----FAY	WGQGTLLVTVSA
VB4B	KATLTADKSSSTAYMEISLTSEDSAVYFCAR	GYDD----YSFAY	WGQGTLLVTVSA
VB51	KATLTADKSSSIAYMQLSSLTSEDSAVYFCTS	GYDD----YSFAY	WGQGTLLVTVSA

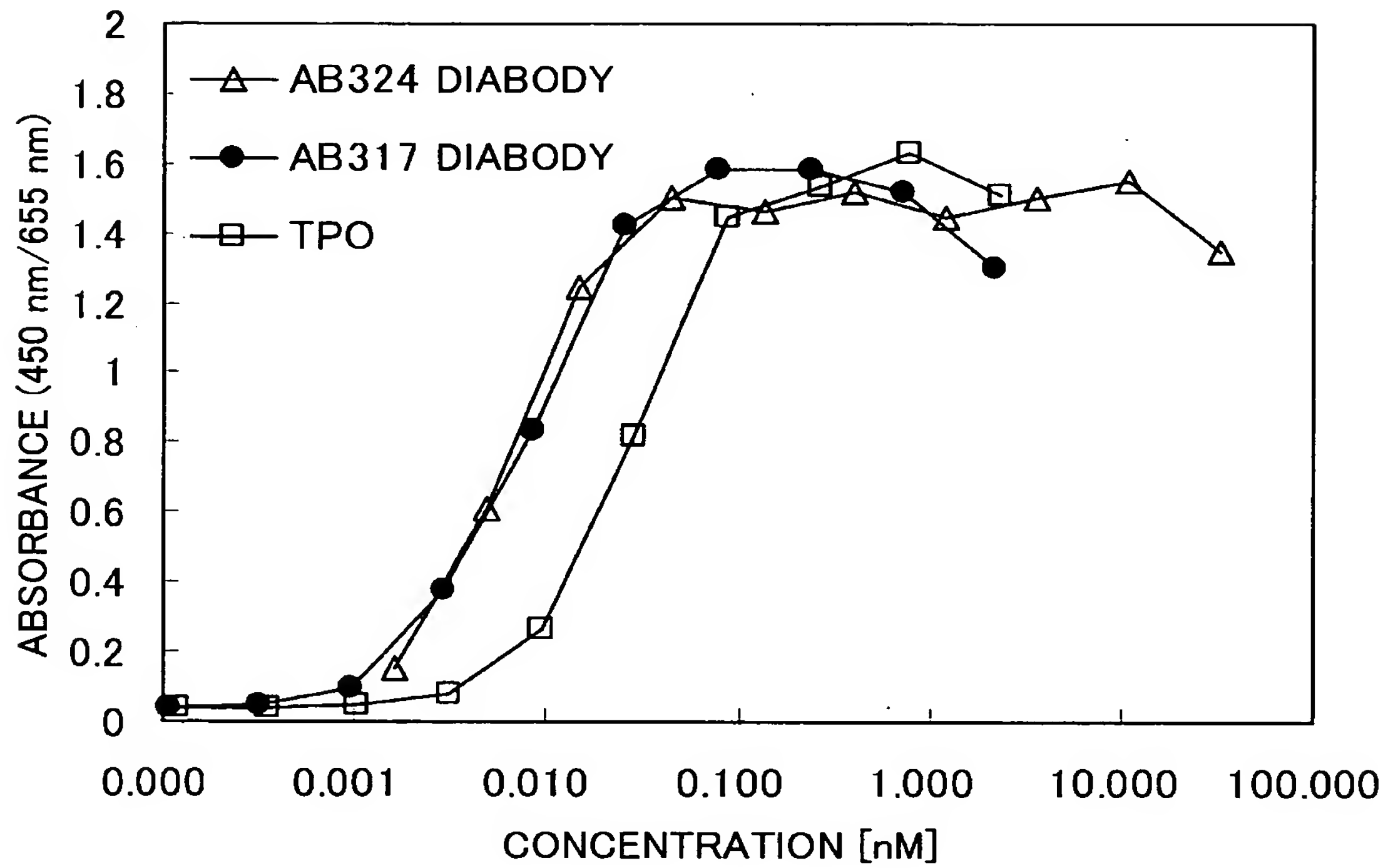
[Fig. 7]

		CDR1	CDR2
VA7	DIVMTQAAPSI PVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY RMSNLAS
VA130	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY RMSNLAS
VA259	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY RMSNLAS
VB17B	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY RMSNLAS
VB12B	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY RMSNLAS
VB140	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY RMSNLAS
VB33	DIVMTQAAPSVPVTPGESVSISC	RSSKSLLYSNGNIYLY	WFLQRPQGSPQLLIY RMSNLAS
VB45B	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY RMSNLAS
VB8B	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFMQRPQGSPQLLIY RMSNLAS
VB115	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY RMSNLAS
VB14B	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY RMSNLAS
VB22B	DIVMTQAAPSI PVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY RMSNLAS
VB16	DIVMTQAAPSVPVTPGESVSISC	RSSKSLLYSNGNTYLY	WFLQRPQGSPQLLIY RMSNLAS
VB157	DIVMTQAAPSVSVTPGESVSISC	RSSKSLLYSNGNIYLY	WFLQRPQGSPQLLIY RMSNLAS
VB4B	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY RMSNLAS
VB51	DIVMTQAAPSLPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY RMSNLAS
		CDR3	
VA7	GVPDRFSGSGSGTAFTLRISRVEAEDVGIYYC	MQHLEYPFT	FGTGTKLEIK
VA130	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VA259	GAPDRFSGSGSGTAFTLRISRVETEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB17B	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB12B	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB140	GVPDRFSGSGSGAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB33	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB45B	GVPDRFSGSGSGAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB8B	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHVEYPYT	FGSGTKLEIK
VB115	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB14B	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB22B	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHIEYPFT	FGSGTKLEIK
VB16	GVPDRFSGSGSGTAFTLTISSVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB157	GVPDRFSGSGSGTAFTLKISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB4B	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHIEYPFT	FGSGTKLEIK
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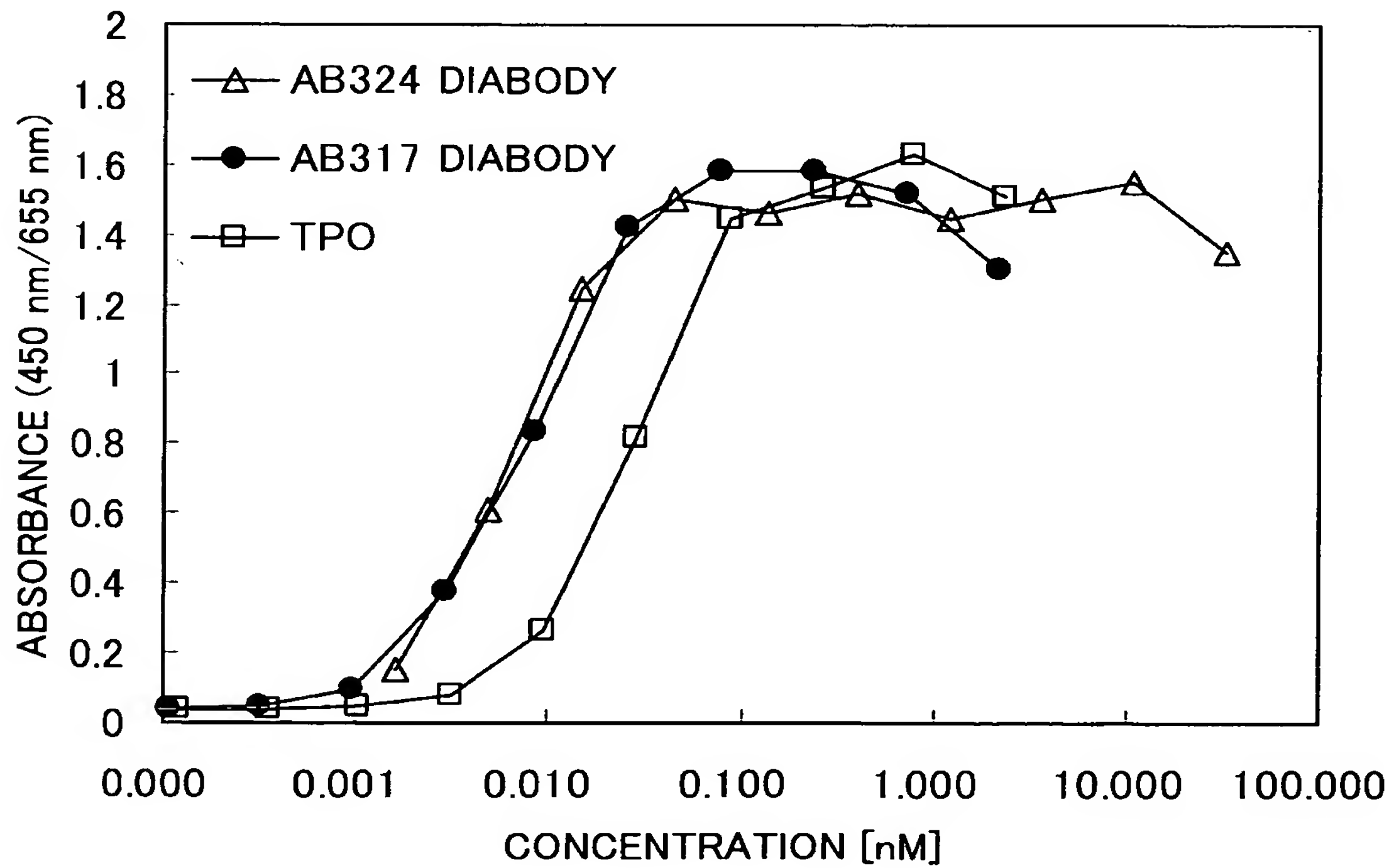
[Fig. 8]



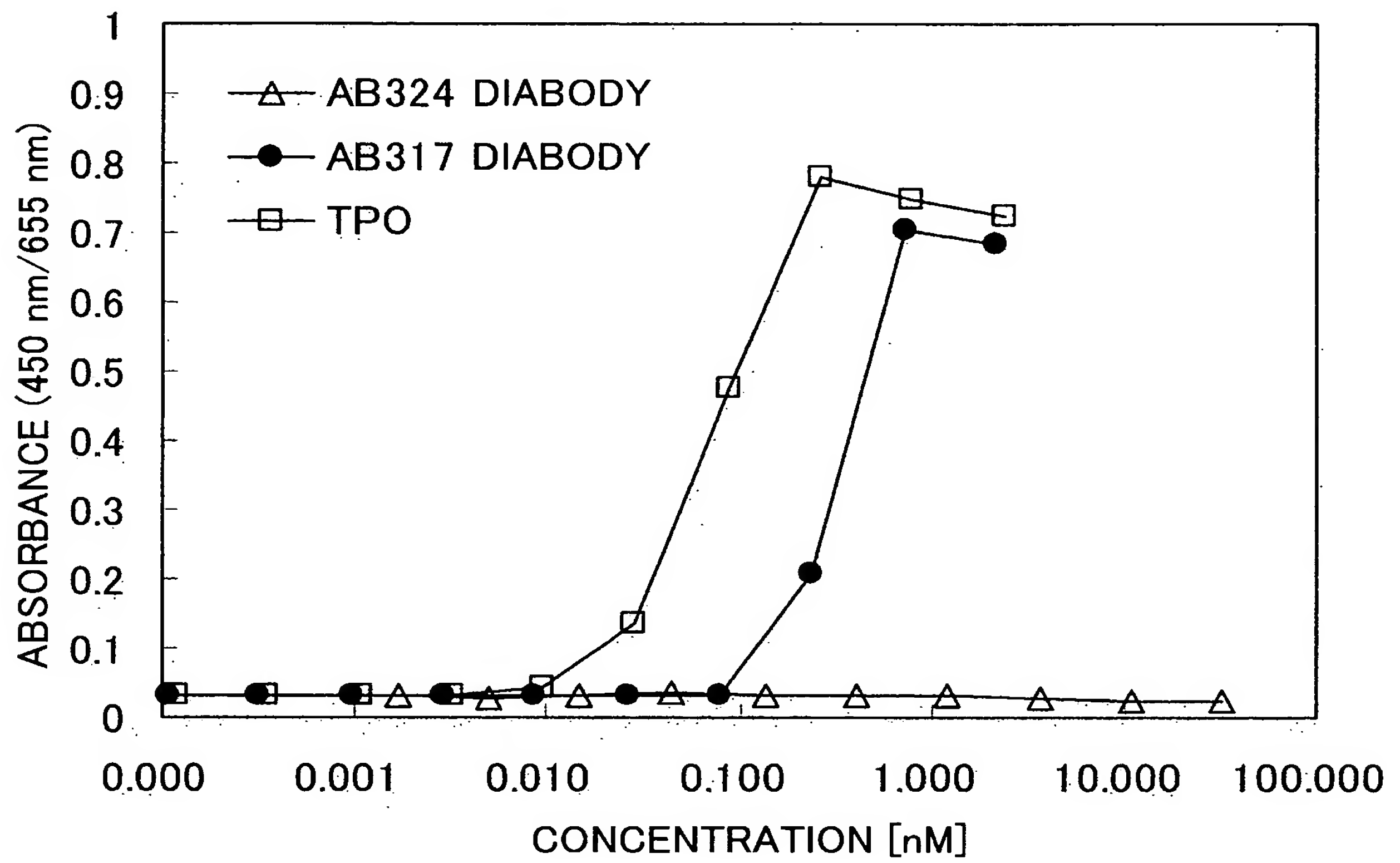
[Fig. 9]



[Fig. 10]



[Fig. 11]



[Document Name] Abstract

[Abstract]

5 [Problems to be Solved] An objective of the present invention is to provide novel anti-Mpl antibodies having TPO-agonistic activity.

[Means for Solving the Problems] Anti-human Mpl antibodies were isolated and purified, and then anti-human Mpl diabodies and anti-human Mpl sv(Fv)₂ were purified using genetic engineering techniques. Furthermore, the present inventors succeeded in humanizing anti-human Mpl sc(Fv)₂.

10 The diabodies and sc(Fv)₂ were assayed for TPO-like agonistic activity, and were found to have activities higher than those of anti-human Mpl antibodies, or activities equivalent to or higher than those of naturally-occurring human TPO ligand.

[Selected Drawings] None